

**Exploring the Membrane Properties of Synthetic
Polymer Vesicles Prepared *via* Ring Opening
Metathesis Polymerisation Induced Self-Assembly**

By

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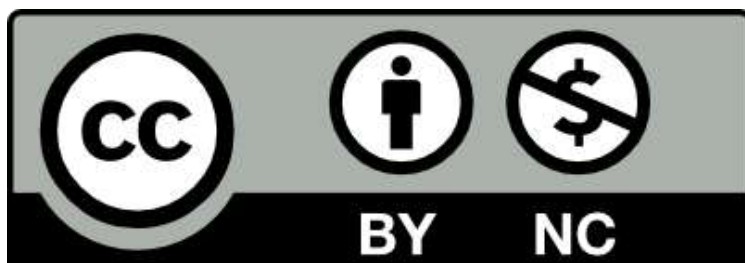
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Abstract

Membrane bound structures are integral to highly complex biological systems due to their compartmentalisation of material. A multitude of highly controlled and efficient membrane transport mechanisms enable biological systems to regulate the passage of a wide range of substrates. Inspired by this, biomimetic structures have been developed to replicate these processes for various applications. Most notably, polymersomes (vesicular structures formed from amphiphilic block copolymers) have been developed and utilised in applications such as drug delivery vehicles and catalytic nanoreactors.

This thesis develops a wider understanding of the properties of block copolymer nano-objects formed by ring-opening metathesis polymerisation induced self-assembly (ROMPISA), in particular their fusion behaviour and ability to encapsulate material. Chapter 1 summarises different polymerisation techniques, polymer self-assembly methods, and particle characterisation techniques. This chapter also provides an overview of the applicability of polymersomes as biomimetic structures. Chapter 2 describes the triggered fusion of carboxylic acid-functionalised polymersomes by a pH switch and how the mechanism of fusion impacts the morphology of the fused structures. Dual, pH-triggered fusion of zwitterionic polymersomes are also explored. Chapter 3 reports the charge-mediated fusion of oppositely charged polymersomes and highlights the effect of size discrepancy on final nano-object morphology. In addition, this chapter determines the mode of fusion for a model ROMPISA system by fluorescence analysis. Chapter 4 examines the encapsulation of water-soluble cargo within ROMPISA nano-objects and how the mechanism of formation largely influences this ability.

This thesis is dedicated to my mum, Sue.

Acknowledgments

So here I am, at the point of writing the acknowledgements for my PhD thesis. I started this journey over four years ago and it really has been a rollercoaster from start to finish. But I would not be here today if it wasn't for several people who have been there with me, every step of the way.

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Table of Contents

Abstract.....	i
Acknowledgments.....	iii
Table of Contents.....	v
List of Figures.....	ix
List of Tables.....	xxvi
List of Schemes	xxx
List of Symbols and Abbreviations	xxxii
Declaration of Authorship.....	xxxviii
Chapter 1. Introduction	1
1.1 Understanding the biological world	2
1.1.1 The role of compartmentalisation in biology	2
1.1.2 Material trafficking in biological structures	3
1.2 Polymers.....	9
1.2.1 Properties of polymers	10
1.2.2 Polymerisation methods.....	14
1.3 Amphiphilic block copolymer self-assembly in solution	19
1.3.1 Polymerisation induced self-assembly	21
1.3.2 Ring-opening metathesis polymerisation induced self-assembly	22
1.3.3 Characterisation techniques for polymer self-assemblies.....	27
1.4 Polymersomes as biomimetic structures	32

1.4.1 Polymersome fusion	33
1.4.2 Encapsulation of cargo	40
1.5 Summary.....	43
1.6 References.....	44
Chapter 2. Expanding the Scope of pH-Triggered Fusion of Polymersomes Synthesised <i>via</i> Aqueous Ring-Opening Metathesis Polymerisation Induced Self-Assembly.....	54
2.1 Abstract.....	55
2.2 Introduction.....	56
2.3 Results and Discussion.....	66
2.3.1 Triggered polymersome fusion of carboxylic acid-containing triblock nano-objects synthesised <i>via</i> ROMPISA	66
2.3.2 Dual pH-responsive particles <i>via</i> ROMPISA	88
2.4 Conclusions.....	101
2.5 Experimental.....	104
2.5.1 Materials.....	104
2.5.2 Characterisation Techniques.....	104
2.5.3 Monomer Synthesis	106
2.5.4 Synthesis and characterisation of carboxylic acid-containing triblock nano-objects <i>via</i> ROMPISA.....	108
2.5.5 Triggered fusion of carboxylic acid-containing triblock nano-objects	118
2.5.6 Synthesis and characterisation of zwitterionic quadrablock nano-objects <i>via</i> ROMPISA	122
2.5.7 Dual pH triggering of zwitterionic nano-objects	126

2.6 References	131
Chapter 3. Investigating Charge-Mediated Fusion of Polymersomes Synthesised <i>via</i> Aqueous Ring-Opening Metathesis Polymerisation Induced Self-Assembly.....	
134	
3.1 Abstract	135
3.2 Introduction	136
3.3 Results and Discussion	142
3.3.1 Synthesis of amine-containing triblock nano-objects <i>via</i> ROMPISA in salt solution	142
3.3.2 Mixing oppositely charged polymersomes synthesised <i>via</i> ROMPISA.....	145
3.3.3 Determination of the Mode of Fusion (hetero- vs homo-fusion).....	151
3.4 Conclusion.....	167
3.5 Experimental	169
3.5.1 Materials	169
3.5.2 Characterisation Techniques	169
3.5.3 Monomer Synthesis.....	172
3.5.4 Mixing oppositely charged nano-objects synthesised <i>via</i> ROMPISA	179
3.5.5 Mode of fusion determination	183
3.6 References	193
Chapter 4. Exploring the Encapsulation of Cargo Within Nano-Objects Synthesised <i>via</i> Aqueous Ring-Opening Metathesis Polymerisation Induced Self-Assembly.....	
197	
4.1 Abstract	198
4.2 Introduction	199

4.2.1 Polymersome encapsulation.....	199
4.2.2 Polymersome mechanism of formation.....	202
4.3 Results and Discussion.....	205
4.3.1 Determining the encapsulation efficiency of a small molecule fluorophore within nano-objects synthesised <i>via</i> aqueous ROMPISA	205
4.3.2 Encapsulation of inorganic nanoparticles into polymeric nano-objects synthesised <i>via</i> aqueous ROMPISA	219
4.3.3 Investigating the mechanism of formation of nano-objects synthesised <i>via</i> aqueous ROMPISA using small angle x-ray scattering.....	226
4.4 Conclusion	237
4.5 Experimental.....	239
4.5.1 Materials.....	239
4.5.2 Characterisation Techniques.....	239
4.5.3 Encapsulation of HPTS within ROMPISA nano-objects	242
4.5.4 Purification of HPTS from nano-objects <i>via</i> ROMPISA.....	242
4.5.5 Concentration dependence of HPTS.....	245
4.5.6 Encapsulation of inorganic NPs within ROMPISA nano-objects	245
4.5.7 Mechanism of Formation	248
4.6 References.....	266
Chapter 5. Conclusions and Future Work.....	271
5.1 References.....	277

List of Figures

Figure 1.1. Schematic illustration of the levels of organisation in multicellular organisms. Adapted from ref. ¹	2
Figure 1.2. Chemical structure of a phospholipid molecule; structural organisation of phospholipids forming a planar bilayer; spontaneous closure of phospholipid bilayer to form a sealed compartment.....	3
Figure 1.3. Mechanisms of passive membrane transport across the phospholipid bilayer.	5
Figure 1.4. Bulk membrane transport across the membrane – exocytosis and endocytosis. Adapted from ref. ²⁰	7
Figure 1.5. Mechanisms of SNARE proteins in membrane fusion. Adapted from Azimi <i>et al.</i> ²⁵ ...	8
Figure 1.6. Schematic diagram of the separation of two distinct polymers with different molecular weights by SEC.....	12
Figure 1.7. Different topologies, compositions, and functionalities of polymers.....	13
Figure 1.8. Increase in polymer molecular weight versus monomer conversion for chain-growth, step-growth, and living polymerisation.....	14
Figure 1.9. Chemical structures of different generations of Grubbs' metathesis catalysts.....	19
Figure 1.10. Amphiphilic block copolymer morphologies in solution. Adapted from Nunes <i>et al.</i> ⁵²	20
Figure 1.11. Schematic representation of polymerisation induced self-assembly (PISA). Adapted from Nunes <i>et al.</i> ⁵²	21

Figure 1.12. One-pot synthesis of shell-functionalised polymeric nanoparticle by ROMPISA. Adapted from Zhang <i>et al.</i> ⁶⁸	23
Figure 1.13. (A) Reaction scheme for the preparation of PNB- <i>b</i> -PA nano-objects by ROMPISA (B) Proposed mechanism for the <i>in situ</i> formation of PNB- <i>b</i> -PA into nanocaterpillars and transmission electron microscopy image of the final morphology. Adapted from Yoon <i>et al.</i> ⁶⁹	24
Figure 1.14. (A) Reaction scheme for the formation of peptide-functionalised diblock copolymers using AquaMet initiator. (B) Transmission electron microscopy images for peptide-functionalised nano-objects before and after incubation with thermolysin. Adapted from Wright <i>et al.</i> ⁷²	25
Figure 1.15. (A) Reaction scheme for the formation of nano-objects by aqueous ROMPISA using a macroinitiator approach. (B) Dry-state and cryo-transmission electron microscopy images of the different morphologies formed. Adapted from Foster <i>et al.</i> ⁷⁵	26
Figure 1.16. Schematic representation of the staining procedure in TEM sample preparation.....	29
Figure 1.17. Schematic representation of liposomes (left) versus polymersomes (right). The key properties offered by each structure are shown in the centre, Adapted from Messenger <i>et al.</i> ⁹³	33
Figure 1.18. Hypothesised energy diagram for the three types of polymersome fusion: (1) spontaneous, (2) induced, and (3) accelerated.....	35

Figure 1.19. (A) Schematic representation of tubesome evolution <i>via</i> polymerisation-induced polymersome fusion. (B) Dry-state TEM images of the various morphologies formed at different core DPs. Adapted from Varlas <i>et al.</i> ¹¹⁵	37
Figure 1.20. (A) Schematic representation of content mixing assay utilising the proton pump <i>bo3</i> oxidase (<i>bo3</i>) and the proton gradient consumer ATP synthase (AS). (B) Comparison of ATP synthesis rates after SNARE-free or SNARE-mediated fusion of different vesicles. Adapted from Otrin <i>et al.</i> ¹²¹	40
Figure 1.21. Schematic visualisation of possible locations of encapsulated cargo within the polymersome: inner (3) and outer (1) hydrophilic surfaces, the hydrophobic membrane (2) or the lumen (4). Adapted from Gumz <i>et al.</i> ¹²⁵	41
Figure 2.1. Schematic representation of a SNARE-mediated membrane fusion process. Adapted from Chen <i>et al.</i> ⁷	57
Figure 2.2. Chemical structures of barbituric acid (BAR) and 2,4,6-triaminopyrimidine (TAP) and their associated hydrogen bonding interactions	58
Figure 2.3. (A) Chemical structure of two β -diketone ligands forming a complex with an Eu^{3+} ion. (B) Schematic diagram of the fusion process between two β -diketone-functionalised liposomes when triggered with EuCl_3 . Adapted from Haluska <i>et al.</i> ¹³	59
Figure 2.4. (A) Reaction scheme for the formation of a triazole-bridged phospholipid by a Cu(I)-catalysed Huisgen–Sharpless click reaction between alkyne- and azide-functionalised phospholipids. (B) Proposed two-step mechanism for the Cu click-mediated fusion of liposomes. Adapted from Loosli <i>et al.</i> ¹⁴	60

Figure 2.5. Schematic diagram of triggered polymersome fusion of triblock polymersomes by a pH switch and TEM images of the resulting nano-objects. Adapted from Fielden <i>et al.</i> ²⁰	63
Figure 2.6. Chemical structures of monomers and initiator used in this chapter.....	66
Figure 2.7. Normalised SEC traces (THF eluent, PMMA standards) of P_{pH2}1_x and intermediate polymers.....	69
Figure 2.8. Normalised DLS analysis of P_{pH2}1_x particles: x = 100, 200, and 300.....	70
Figure 2.9. Dry-state TEM images of P_{pH2}1_x particles: x = 100, 200, and 300. Scale bar = 200 nm.....	70
Figure 2.10. Normalised DLS analysis of P_{pHy}1₂₀₀ particles synthesised at pH 2, 3, 4.5, and 7....	72
Figure 2.11. Dry-state TEM images of P_{pHy}1₂₀₀ particles synthesised at pH 2, 3, 4.5, and 7. Scale bar = 200 nm.....	73
Figure 2.12. Normalised SEC traces (THF eluent, PMMA standards) of P1_x and intermediate polymers.....	75
Figure 2.13. Normalised DLS analysis of P1_x particles: x = 100, 200, and 300.....	75
Figure 2.14. Dry-state TEM images of P1_x particles: x = 100, 200, and 300. Scale bar = 200 nm.....	76
Figure 2.15. (A) Average size of particles acidified by different concentrations of acid, as determined by dry-state TEM analysis. Dry-state TEM images of P1₂₀₀ particles synthesised at pH 7 and acidified with different concentrations of HCl solution (+ 10% THF): 5, 10, 25, 50, and 100 mM. Scale bar = 200 nm.....	78

Figure 2.16. Hypothesised energy diagram of the first step in pH-triggered fusion of triblock nano-objects containing carboxylic acid groups that are either (A) partially protonated (dashed) or (B) fully protonated (solid).....	79
Figure 2.17. Normalised DLS analysis of P1_x particles acidified to pH 2: x = 100, 200, and 300.....	80
Figure 2.18. Dry-state TEM images of P1_x particles synthesised at pH 7 and acidified to pH 2: x = 100, 200, and 300. Scale bar = 200 nm.....	81
Figure 2.19. Average size of P1_x particles synthesised at pH 7 (light blue) and acidified to pH 2 (dark blue), as determined by dry-state TEM.....	81
Figure 2.20. Normalised DLS analysis of P1₂₀₀ particles synthesised at pH 7 and acidified to pH 2, analysed at different time points: 15, 30, 60, 120, and 300 minutes.....	83
Figure 2.21. (A) Average size of particles synthesised at pH 7 and acidified to pH 2, analysed at different time points, as determined by dry-state TEM analysis. Dry-state TEM images of P1₂₀₀ particles synthesised at pH 7 and acidified to pH 2, analysed at different time points: 15, 30, 60, 120, and 300 minutes. Scale bar = 200 nm.....	84
Figure 2.22. Hypothesised energy diagram for the two-step pH-triggered fusion of triblock nano-objects containing either amine (red solid) or carboxylic acid groups (blue dashed).....	85
Figure 2.23. Schematic showing the two-step fusion process and their coronal overlap for triblock nano-objects of different sizes.....	86
Figure 2.24. Suggested fusion mechanism when the rate of adhesion is (A) greater than or (B) less than the rate of chain rearrangement.....	87

Figure 2.25. Schematic diagram showing the different block orders and microstructures targeted for the polymers in each dual pH-responsive particle.....	88
Figure 2.26. Normalised SEC traces (THF eluent, PMMA standards) of P2₂₀₀ and intermediate polymers.....	90
Figure 2.27. Normalised SEC traces (THF eluent, PMMA standards) of P3₂₀₀ and intermediate polymers.....	91
Figure 2.28. Normalised SEC traces (THF eluent, PMMA standards) of P4₂₀₀ and intermediate polymers.....	91
Figure 2.29. Normalised DLS analysis of P2₂₀₀ , P3₂₀₀ , and P4₂₀₀	92
Figure 2.30. Dry-state TEM images of P2₂₀₀ , P3₂₀₀ , and P4₂₀₀ . Scale bar = 200 nm.....	92
Figure 2.31. Dry-state TEM images of P2₂₀₀ , P3₂₀₀ and P4₂₀₀ particles synthesised at pH 7, acidified to pH 2 and then basified to pH 12.....	94
Figure 2.32. Dry-state TEM images of P2₂₀₀ , P3₂₀₀ and P4₂₀₀ particles synthesised at pH 7, basified to pH 12 and then acidified to pH 2.....	97
Figure 2.33. Average particle size, as determined by dry-state TEM, for P2₂₀₀ (blue squares), P3₂₀₀ (green circles), and P4₂₀₀ (orange triangles) particles switched to (A) pH 2 then 12 and (B) pH 12 then 2. (C) Increase in particle size for each pH switch step for P2₂₀₀ , P3₂₀₀ and P4₂₀₀ particles.....	98
Figure S2.1. ¹ H NMR spectrum (400 MHz, CDCl ₃ , 298 K) of NB-COOH	107
Figure S2.2. ¹³ C NMR spectrum (400 MHz, CDCl ₃ , 298 K) of NB-COOH	108

Figure S2.3. Inverted vial test of $P_{pH2}1_x$, from left to right $x = 100, 200$ and 300	109
Figure S2.4. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P(NB-PEG)_{11} + THF$	110
Figure S2.5. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P(NB-PEG)_{11}-b-P(NB-COOH)_5 + THF$	110
Figure S2.6. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P_{pH2}1_{100} + THF/PB2$	111
Figure S2.7. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P_{pH2}1_{200} + THF/PB2$	111
Figure S2.8. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P_{pH2}1_{300} + THF/PB2$	112
Figure S2.9. Supplementary dry-state TEM images of $P_{pH2}1_x$ particles: $x = 100, 200$, and 300 . Scale bar = 100 nm.....	113
Figure S2.10. Dry-state TEM image analysis results for $P_{pH2}1_{100}$ particles, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.....	113
Figure S2.11. Normalised SEC traces (THF eluent, PMMA standards) of $P_{pHy}1_{200}$	114
Figure S2.12. Supplementary dry-state TEM images of $P_{pHy}1_{200}$ particles synthesised at pH 2, 3, 4.5, and 7. Scale bar = 100 nm.....	114
Figure S2.13 Dry-state TEM image analysis results for $P_{pHy}1_{200}$ particles: pH 4.5 and 7, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.....	115
Figure S2.14. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P1_{100} + THF/Aq_{NaCl}$	116
Figure S2.15. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P1_{200} + THF/Aq_{NaCl}$	116
Figure S2.16. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P1_{300} + THF/Aq_{NaCl}$	117

Figure S2.17. Supplementary dry-state TEM images of P1_x particles: x = 100, 200, and 300. Scale bar = 100 nm.....	117
Figure S2.18. Dry-state TEM image analysis results for P1_x particles: x = 100, 200, and 300, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.....	118
Figure S2.19. Normalised DLS analysis of P1₂₀₀ particles acidified with different concentrations of HCl solution (+ 10% THF): 5, 10, 25, 50, and 100 mM.....	119
Figure S2.20. Supplementary dry-state TEM images of P1_x particles synthesised at pH 7 and acidified to pH 2: x = 100, 200, and 300. Scale bar = 100 nm.....	120
Figure S2.21. Dry-state TEM image analysis results for P1₁₀₀ particles acidified to pH 2, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.....	120
Figure S2.22. Supplementary dry-state TEM images of P1₂₀₀ particles synthesised at pH 7 and acidified to pH 2, analysed at different time points: 15, 30, 60, 120, and 300 minutes. Scale bar = 100 nm.....	121
Figure S2.23. Supplementary dry-state TEM images of P2₂₀₀ , P3₂₀₀ , and P4₂₀₀ . Scale bar = 100 nm.....	124
Figure S2.24. Dry-state TEM image analysis results for P2₂₀₀ , P3₂₀₀ , and P4₂₀₀ , as determined by image processing using ImageJ analysis software.....	125
Figure S2.25. Zeta potential values for P2₂₀₀ (blue), P3₂₀₀ (green), and P4₂₀₀ (orange) synthesised at pH 7, measured from microelectrophoretic analysis.....	126

Figure S2.26. Normalised DLS analysis of P2₂₀₀ , P3₂₀₀ , and P4₂₀₀ particles acidified to pH 2 and then switched to pH 12.....	127
Figure S2.27. Supplementary dry-state TEM images of P2₂₀₀ , P3₂₀₀ and P4₂₀₀ particles synthesised at pH 7, acidified to pH 2 and then basified to pH 12. Scale bar = 100 nm.....	128
Figure S2.28. Normalised DLS analysis of P2₂₀₀ , P3₂₀₀ , and P4₂₀₀ particles basified to pH 12 and then switched to pH 2.....	129
Figure S2.29. Supplementary dry-state TEM images of P2₂₀₀ , P3₂₀₀ and P4₂₀₀ particles synthesised at pH 7, basified to pH 12 and then acidified to pH 2. Scale bar = 100 nm.....	130
Figure 3.1. Mechanisms of membrane fusion in biological systems. Adapted from Martens <i>et al.</i> ¹	136
Figure 3.2 Schematic diagram of a three-stage liposome-fusion cascade encoded by three sets of complementary membrane-anchored DNA strands. Adapted from Löffler <i>et al.</i> ¹¹	138
Figure 3.3. Charge-mediated membrane mixing of hybrid large unilamellar vesicles. Three combinations were tested: anionic/cationic, neutral/cationic, and anionic/neutral vesicles. Adapted from Marušič <i>et al.</i> ³³	140
Figure 3.4. Schematic diagram of charge-mediated fusion between oppositely charged triblock polymersomes prepared <i>via</i> ROMPISA.....	140
Figure 3.5. Normalised SEC traces (THF eluent, PMMA standards) of P2_y and intermediate polymers.....	143

Figure 3.6. Normalised DLS analysis of P2_y particles: $y = 100, 200,$ and 300	144
Figure 3.7. Dry-state TEM images of P2_y particles: $y = 100, 200,$ and 300 . Scale bar = 200 nm.....	144
Figure 3.8. Dry-state TEM images of P1_x ($x = 100, 200,$ and 300) and P2_y ($y = 100, 200,$ and 300) particles and their corresponding mixtures (P1_x + P2_y) at the intersection. Scale bar = 200 nm.....	146
Figure 3.9. Suggested fusion mechanisms for (A) unilateral fusion and (B) cluster fusion.	147
Figure 3.10. Average particles size, as determined by dry-state TEM, compared to the core DP for the constituent P1_x and P2_y particles.....	149
Figure 3.11. Suggested packing structures for fused membranes in P1_x + P2_y mixtures with either (1) equivalent core DPs or (2) inequivalent core DPs between particles.....	150
Figure 3.12. Proposed modes of fusion resulting from the mixing of two different particles.....	151
Figure 3.13. Dry-state TEM images of P3₁₀₀ , P4₂₀₀ , and P3₁₀₀ + P4₂₀₀ . Scale bar = 200 nm.....	154
Figure 3.14. Chemical structures for fluorescent ROMPISA monomers NB-Pyr and NB-NI	155
Figure 3.15. Normalised UV-vis absorption spectra (dotted lines) of NB-Pyr (green) and NB-NI (purple). Normalised fluorescence emission spectra (solid lines) of NB-Pyr ($\lambda_{\text{Ex}} = 347$ nm, green) and NB-NI ($\lambda_{\text{Ex}} = 405$ nm, purple).....	156

Figure 3.16. Schematic diagram showing the different monomers and microstructures targeted for the polymers in each fluorescently labelled particle.....	157
Figure 3.17. Normalised SEC traces (THF eluent, PMMA standards) of P3-Pyr₁₀₀ and intermediate polymers. RI (solid line), UV 340 (dotted line) and UV 400 nm (dashed line).....	158
Figure 3.18. Normalised SEC traces (THF eluent, PMMA standards) of P4-NI₁₀₀ and intermediate polymers. RI (solid line), UV 340 (dotted line) and UV 400 nm (dashed line).....	159
Figure 3.19. Normalised SEC traces (THF eluent, PMMA standards) of P4-NI₂₀₀ and intermediate polymers. RI (solid line), UV 340 (dotted line) and UV 400 nm (dashed line).....	159
Figure 3.20. Normalised SEC traces (THF eluent, PMMA standards) of P4-Pyr-NI₂₀₀ and intermediate polymers. RI (solid line), UV 340 (dotted line) and UV 400 nm (dashed line).....	160
Figure 3.21. Dry-state TEM images of (A) P3-Pyr₁₀₀ , (B) P4-NI₁₀₀ , (C) P4-NI₂₀₀ , (D) P4-Pyr-NI₂₀₀ . Scale bar = 200 nm.....	160
Figure 3.22. Normalised fluorescence excitation (dotted lines) and emission (solid lines) spectra of (A) P3-Pyr₁₀₀ , (B) P4-NI₁₀₀ , (C) P4-NI₂₀₀ and (D) P4-Pyr-NI₂₀₀ . Green lines refer to NB-Pyr fluorescence ($\lambda_{Ex} = 347$, $\lambda_{Em} = 377$ nm), purple lines refer to NB-NI fluorescence ($\lambda_{Ex} = 405$, $\lambda_{Em} = 512$ nm), orange lines refer to NB-NI fluorescence ($\lambda_{Ex} = 347$, $\lambda_{Em} = 512$ nm).....	162
Figure 3.23. Dry-state TEM images of (A) P3₁₀₀ + P4₂₀₀ , (B) P3₁₀₀ + P4-NI₂₀₀ , (C) P3₁₀₀ + P4-Pyr-NI₂₀₀ , (D) P3-Pyr₁₀₀ + P4₂₀₀ , (E) P3-Pyr₁₀₀ + P4-NI₁₀₀ , (F) P3-Pyr₁₀₀ + P4-NI₂₀₀ . Scale bar = 200 nm.....	164

Figure 3.24. Normalised fluorescence emission spectra of P3-Pyr ₁₀₀ + P4-NI ₁₀₀ (black line) and P3-Pyr ₁₀₀ + P4-NI ₂₀₀ (red line). Fluorescence emission at $\lambda_{\text{Ex}} = 347$ nm (solid lines) was normalised to the emission intensity maximum at $\lambda_{\text{Ex}} = 405$ nm (dashed lines).....	165
Figure S3.1. ¹ H NMR spectrum (400 MHz, CDCl ₃ , 298 K) of NB-Pyr	173
Figure S3.2. ¹³ C NMR spectrum (101 MHz, CDCl ₃ , 298 K) of NB-Pyr	173
Figure S3.3. ¹ H NMR spectrum (400 MHz, (CH ₃) ₂ SO ₄ -d ₆ , 298 K) of intermediate 2	174
Figure S3.4. ¹ H NMR spectrum (400 MHz, CDCl ₃ , 298 K) of intermediate 3	176
Figure S3.5. ¹³ C NMR spectrum (101 MHz, CDCl ₃ , 298 K) of intermediate 3	176
Figure S3.6. ¹ H NMR spectrum (400 MHz, CDCl ₃ , 298 K) of NB-NI	178
Figure S3.7. ¹³ C NMR spectrum (400 MHz, CDCl ₃ , 298 K) of NB-NI	178
Figure S3.8. Supplementary dry-state TEM images of P2_y particles: y = 100, 200, and 300. Scale bar = 100 nm.....	179
Figure S3.9. Dry-state TEM image analysis results for P2_y particles synthesised at pH 7: y = 100, 200, and 300, as determined by image processing using ImageJ analysis software.....	180
Figure S3.10. Normalised DLS analysis of (A) P1 ₁₀₀ + P2 ₁₀₀ , (B) P1 ₁₀₀ + P2 ₂₀₀ , (C) P1 ₁₀₀ + P2 ₃₀₀ , (D) P1 ₂₀₀ + P2 ₁₀₀ , (E) P1 ₂₀₀ + P2 ₂₀₀ , (F) P1 ₂₀₀ + P2 ₃₀₀ , (G) P1 ₃₀₀ + P2 ₁₀₀ , (H) P1 ₃₀₀ + P2 ₂₀₀ and (I) P1 ₃₀₀ + P2 ₃₀₀	181
Figure S3.11. Supplementary dry-state TEM images of (A) P1 ₁₀₀ + P2 ₁₀₀ , (B) P1 ₁₀₀ + P2 ₂₀₀ , (C) P1 ₁₀₀ + P2 ₃₀₀ , (D) P1 ₂₀₀ + P2 ₁₀₀ , (E) P1 ₂₀₀ + P2 ₂₀₀ , (F) P1 ₂₀₀ + P2 ₃₀₀ , (G) P1 ₃₀₀ + P2 ₁₀₀ , (H) P1 ₃₀₀ + P2 ₂₀₀ and (I) P1 ₃₀₀ + P2 ₃₀₀	182

Figure S3.12. Average particle size of P1_x (blue), P2_y (red) and P1_x + P2_y (black), as determined by dry-state TEM analysis.....	183
Figure S3.13. Normalised SEC traces (THF eluent, PMMA standards) of (A) P3₁₀₀ and (B) P4₂₀₀ and intermediate polymers.....	186
Figure S3.14. Normalised DLS analysis of (A) P3₁₀₀ , (B) P4₂₀₀ , (C) P3-Pyr₁₀₀ , (D) P4-NI₁₀₀ , (E) P4-NI₂₀₀ and (F) P4-Pyr-NI₂₀₀	187
Figure S3.15. Supplementary dry-state TEM images of (A) P3₁₀₀ , (B) P4₂₀₀ , (C) P3-Pyr₁₀₀ , (D) P4-NI₁₀₀ , (E) P4-NI₂₀₀ , (F) P4-Pyr-NI₂₀₀ . Scale bar = 100 nm.....	189
Figure S3.16. Normalised DLS analysis of (A) P3₁₀₀ + P4₂₀₀ , (B) P3₁₀₀ + P4-NI₂₀₀ , (C) P3₁₀₀ + P4-Pyr-NI₂₀₀ , (D) P3-Pyr₁₀₀ + P4₂₀₀ , (E) P3-Pyr₁₀₀ + P4-NI₁₀₀ and (F) P3-Pyr₁₀₀ + P4-NI₂₀₀	190
Figure S3.17. Supplementary dry-state TEM images of (A) P3₁₀₀ + P4₂₀₀ , (B) P3₁₀₀ + P4-NI₂₀₀ , (C) P3₁₀₀ + P4-Pyr-NI₂₀₀ , (D) P3-Pyr₁₀₀ + P4₂₀₀ , (E) P3-Pyr₁₀₀ + P4-NI₁₀₀ and (F) P3-Pyr₁₀₀ + P4-NI₂₀₀	191
Figure S3.18. Normalised fluorescence emission spectra of (A) P3₁₀₀ + P4-NI₂₀₀ , (B) P3₁₀₀ + P4-Pyr-NI₂₀₀ and (C) P3-Pyr₁₀₀ + P4₂₀₀ . Green lines refer to NB-Pyr fluorescence ($\lambda_{\text{Ex}} = 347 \text{ nm}$), purple lines refer to NB-NI fluorescence ($\lambda_{\text{Ex}} = 405 \text{ nm}$), and orange lines refer to NB-NI fluorescence ($\lambda_{\text{Ex}} = 347 \text{ nm}$).....	192
Figure 4.1. Schematic diagram of pH-sensitive BODIPY encapsulated within the membrane core of PEG- <i>b</i> -PCL polymersomes and its fluorescence switch on upon a decrease in pH. Adapted from Quan <i>et al.</i> ⁶	200

Figure 4.2. DASA-functionalized visible light-responsive nanoreactors. Adapted from Rifaie-Graham <i>et al.</i> ⁸	201
Figure 4.3. Schematic illustration of the co-delivery system using biodegradable polymersomes to deliver siRNA and DOX. Adapted by Kim <i>et al.</i> ¹⁰	202
Figure 4.4. Schematic representation of the two proposed mechanisms for vesicle formation: mechanism I and mechanism II. Adapted from Iqbal <i>et al.</i> ⁹	203
Figure 4.5. (A) Normalised fluorescence emission spectra of 10 mM HPTS at different solution pH. (B) pH calibration curve for 10 mM HPTS.....	206
Figure 4.6. Normalised SEC traces (THF eluent, PMMA standards) of P1₁₂₀ , with (red) and without (black) HPTS, and intermediate polymers.....	207
Figure 4.7. Normalised SEC traces (THF eluent, PMMA standards) of P2₁₂₀ , with (blue) and without (black) HPTS, and intermediate polymers.....	209
Figure 4.8. Normalised DLS analysis of P2₁₂₀ (A) without and (B) with HPTS.....	210
Figure 4.9. Dry-state TEM images of P2₁₂₀ nano-objects (A) without and (B) with HPTS. Scale bar = 200 nm.....	210
Figure 4.10. Normalised fluorescence emission spectra of P2₁₂₀ before (black) and after (red) dialysis against H ₂ O.....	211
Figure 4.11. Normalised fluorescence emission spectra of P2₁₂₀ before (black) and after (red) dialysis at pH 2 and P2₁₂₀ after being disassembled and reformed (blue).....	212

Figure 4.12. Normalised DLS analysis of P2₁₂₀ (A) before and (B) after purification by gel filtration chromatography.....	214
Figure 4.13. Dry-state TEM images of P2₁₂₀ nano-objects (A) before and (B) after purification by gel filtration chromatography. Scale bar = 200 nm.....	214
Figure 4.14. Normalised fluorescence emission spectra of P2₁₂₀ before (black) and after (red) purification by gel filtration chromatography.....	215
Figure 4.15. Normalised DLS analysis of P2₁₂₀ synthesised at different concentrations of HPTS (A) 0, (B) 1, (C) 10 and (D) 100 mg mL ⁻¹	216
Figure 4.16. Normalised fluorescence emission spectra of P2₁₂₀ synthesised at different concentrations of HPTS (A) 0, (B) 1, (C) 10 and (D) 100 mg mL ⁻¹	217
Figure 4.17. Normalised fluorescence emission spectra of P2₁₂₀ synthesised at different concentrations of HPTS following dialysis at pH 2 (A) 0, (B) 1, (C) 10 and (D) 100 mg mL ⁻¹	218
Figure 4.18. Normalised SEC traces (THF eluent, PMMA standards) of P3₁₈₀ , with (blue) and without (black) silica NPs, and intermediate polymers.....	221
Figure 4.19. Dry-state TEM images of native P3₁₈₀ particles (UA staining); P3₁₈₀ particles synthesised in the presence of 1 wt% silica (1), 2 wt% silica (2), and without silica present but added after self-assembly (1A) (no staining). Scale bar = 200 nm.....	222
Figure 4.20. Normalised DLS analysis of native P3₁₈₀ particles; P3₁₈₀ particles synthesised in the presence of different concentrations of gold NP (0.01 refers to the optical density (OD) of gold NP in solution); and gold nanoparticles (OD = 0.5).....	224

Figure 4.21. Dry-state TEM images of native P3₁₈₀ particles (UA staining); P3₁₈₀ particles synthesised in the presence of gold NP (OD = 0.5), and Gold nanoparticles (OD = 0.5) (no staining). Scale bar = 200 nm.....	225
Figure 4.22. Normalised SEC traces (THF eluent, PMMA standards) of (A) P1_x and (B) P2_x , and intermediate polymers.....	229
Figure 4.23. Normalised DLS analysis of P1_x and P2_x	230
Figure 4.24. Staggered SAXS patterns of (A) P1_x and (B) P2_x fitted to a spherical micelle model (black line). Core DP dependent trends in (C) core radius, (D) aggregation number and (E) particle diameter for P1_x (red) and P2_x (blue) obtained by SAXS analysis.....	231
Figure 4.25. Overlaid (left) and staggered (right) SAXS patterns for (A) P1₄₀ and (B) P2₄₀	232
Figure 4.26. Overlaid SAXS patterns of static (black) and <i>in situ</i> endpoints (red or blue) measurements for (A) P1₂₀ , (B) P2₂₀ , (C) P1₄₀ , (D) P2₄₀ , (E) P1₆₀ and (F) P2₆₀	234
Figure 4.27. Evolution of core radius, aggregation number and particle size as a function of time for (A) P1_x and (B) P2_x nano-objects, as determined by modelling scattering patterns obtained by <i>in situ</i> SAXS analysis. Error bars indicate the standard deviation of the core radius.....	236
Figure S4.1. Normalised DLS analysis of P1₁₂₀ (A) without and (B) with HPTS.....	242
Figure S4.2. Normalised DLS analysis of P2₁₂₀ after dialysis at pH 2.....	243
Figure S4.3. Dry-state TEM image of P2₁₂₀ nano-objects after dialysis at pH 2. Scale bar = 200 nm.....	243

Figure S4.4. Normalised DLS analysis of P2₁₂₀ after purification by spin filtration.....	244
Figure S4.5. Normalised DLS analysis of silica NPs (1 wt %).	246
Figure S4.6. Dry-state TEM image of silica NPs (1 wt %). Scale bar = 200 nm.....	246
Figure S4.7. Normalised DLS analysis of native P3₁₈₀ particles; P3₁₈₀ particles synthesised in the presence of 1 wt% silica (1), 2 wt% silica (2), and without silica present but added after self-assembly (1A).....	247
Figure S4.8. Overlaid (left) and staggered (right) SAXS patterns for (A) P1₂₀ and (B) P2₂₀	252
Figure S4.9. Overlaid (left) and staggered (right) SAXS patterns for (A) P1₆₀ and (B) P2₆₀	253

List of Tables

Table 2.1. Characterisation summary of P_{pH2}1_x	69
Table 2.2. Characterisation summary of P_{pHy}1₂₀₀	71
Table 2.3. Characterisation summary of P1_x	74
Table 2.4. Characterisation summary of P1₂₀₀ particles acidified with different concentrations of HCl.....	77
Table 2.5. Characterisation summary of P1_x particles acidified to pH 2.....	80
Table 2.6. Characterisation summary of P1_x particles acidified to pH 2 at different time points.....	83
Table 2.7. Characterisation summary of P2₂₀₀ , P3₂₀₀ and P4₂₀₀	90
Table 2.8. Characterisation summary of P2₂₀₀ , P3₂₀₀ and P4₂₀₀ acidified to pH 2 then switched pH 12.....	93
Table 2.9 Characterisation summary of P2₂₀₀ , P3₂₀₀ and P4₂₀₀ basified to pH 12 then switched pH 2.....	95
Table S2.1. SEC data of intermediate polymers for P_{pH2}1_x	112
Table S2.2. Polymer composition of zwitterionic quadrablock nano-objects synthesised <i>via</i> ROMPISA.....	123
Table S2.3. SEC data of intermediate polymers for P2₂₀₀ , P3₂₀₀ and P4₂₀₀	124
Table 3.1. Characterisation summary of P2_y	143

Table 3.2. Characterisation summary for P1_x + P2_y mixtures.....	145
Table 3.3. Characterisation summary of P3₁₀₀, P4₂₀₀, and P3₁₀₀ + P4₂₀₀	154
Table 3.4. Property information for fluorescent ROMPISA particles and the relevant control particles.....	157
Table 3.5. Characterisation summary of P3-Pyr₁₀₀, P4-NI₁₀₀, P4-NI₂₀₀ and P4-Pyr-NI₂₀₀	158
Table 3.6. Characterisation summary for P3 + P4 mixtures.....	163
Table S3.1. SEC data of intermediate polymers for P2_y	179
Table S3.2. SEC data of intermediate polymers for P3 and P4	185
Table S3.3. Formulation details for mixing experiments.....	189
Table 4.1. Characterisation summary of P1₁₂₀	207
Table 4.2. Characterisation summary of P2₁₂₀	209
Table 4.3. Characterisation summary of P2₁₂₀ synthesised with different concentrations of HPTS.....	216
Table 4.4. Characterisation summary of P1_x	228
Table 4.5. Characterisation summary of P2_x	228
Table S4.1. Characterisation summary of P2₁₂₀ after dialysis at pH 2.....	242
Table S4.2. Characterisation summary of P2₁₂₀ after spin filtration.....	244
Table S4.3. Characterisation summary of P2₁₂₀ after gel filtration chromatography.....	245

Table S4.4. Fluorescence analysis of P2₁₂₀ synthesised with different concentrations of HPTS before and after dialysis.....	245
Table S4.5. Characterisation summary of P3₁₈₀	245
Table S4.6. Characterisation summary of silica NPs.....	246
Table S4.7. Characterisation summary of P3₁₈₀ synthesised with silica NPs.....	247
Table S4.8. Characterisation summary of gold NPs.....	248
Table S4.9. Characterisation summary of P3₁₈₀ synthesised with gold NPs.....	248
Table S4.10. Summary of variable fitting parameters for P1₂₀ SAXS data.....	254
Table S4.11. Summary of values calculated using SAXS fitting parameters outlined in Table S4.10 for P1₂₀	255
Table S4.12. Summary of variable fitting parameters for P1₄₀ SAXS data.....	256
Table S4.13. Summary of values calculated using SAXS fitting parameters outlined in Table S4.12 for P1₄₀	257
Table S4.14. Summary of variable fitting parameters for P1₆₀ SAXS data.....	258
Table S4.15. Summary of values calculated using SAXS fitting parameters outlined in Table S4.14 for P1₆₀	259
Table S4.16. Summary of variable fitting parameters for P2₂₀ SAXS data.....	260
Table S4.17. Summary of values calculated using SAXS fitting parameters outlined in Table S4.16 for P2₂₀	261

Table S4.18. Summary of variable fitting parameters for P2₄₀ SAXS data.....	262
Table S4.19. Summary of values calculated using SAXS fitting parameters outlined in Table S4.18 for P2₄₀	263
Table S4.20. Summary of variable fitting parameters for P2₆₀ SAXS data.....	264
Table S4.21. Summary of values calculated using SAXS fitting parameters outlined in Table S4.20 for P2₆₀	265

List of Schemes

Scheme 1.1. Generic reaction scheme for alkene metathesis.....	16
Scheme 1.2. Hérissou and Chauvin mechanism for alkene metathesis, showing the formation of the four-membered metallacycle.....	17
Scheme 1.3. Mechanism of ring-opening metathesis polymerisation.....	18
Scheme 2.1. Synthesis of <i>exo</i> -Norbornene imide carboxylic acid (NB-COOH).....	68
Scheme 2.2. Synthetic route for the preparation of PpH21x block copolymers by ROMPISA at pH 2.....	68
Scheme 2.3. Synthetic route for the preparation of P1_x block copolymers by ROMPISA at pH 7.....	74
Scheme S2.1. Synthetic routes for the preparation of P2₂₀₀ , P3₂₀₀ , and P4₂₀₀ quadrablock copolymers by ROMPISA.....	122
Scheme 3.1. Synthetic route for the preparation of P1_x and P2_y triblock copolymers by ROMPISA at pH 7.....	143
Scheme 3.2. Synthetic route for the preparation of P3₁₀₀ and P4₂₀₀ block copolymers by ROMPISA at pH 2.....	153
Scheme S3.1. Synthesis of NB-Pyr	172
Scheme S3.2. Synthesis of intermediate 2	174
Scheme S3.3. Synthesis of intermediate 3 from 2	175
Scheme S3.4. Synthesis of NB-NI from intermediate 3	177

Scheme S3.5. Synthetic route for the preparation of P3-Pyr₁₀₀ block copolymers by ROMPISA.....	183
Scheme S3.6. Synthetic route for the preparation of P4-NI_{100/200} block copolymers by ROMPISA.....	184
Scheme S3.7. Synthetic route for the preparation of P4-Pyr-NI₂₀₀ block copolymers by ROMPISA.....	184
Scheme 4.1. Ionisation equilibrium of HPTS to its phenate form.....	205
Scheme 4.2. Synthesis of P1₁₂₀ diblock copolymer in the presence of HPTS by ROMPISA.....	206
Scheme 4.3. Synthesis of P2₁₂₀ diblock copolymer in the presence of HPTS by ROMPISA.....	208
Scheme 4.4. Synthesis of P3₁₈₀ diblock copolymer in the presence of silica NPs by ROMPISA.....	220
Scheme 4.5. Synthesis of P3₁₈₀ diblock copolymer in the presence of gold NPs by ROMPISA.....	223
Scheme 4.6. Synthesis of P1_x and P2_x diblock copolymers by ROMPISA.....	228

List of Symbols and Abbreviations

% v/v	Volume percentages of two components
°	Degrees
°C	Degrees Celsius
μ_e	Electrophoretic mobility
μL	Microlitre(s)
μm	Micrometre(s)
μM	Micromolar
μmol	Micromole(s)
μs	Microsecond(s)
1D	One-dimension
^1H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
ADP	Adenosine diphosphate
Aq_{NaCl}	Solution of NaCl in H_2O
ATP	Adenosine triphosphate
ATRP	Atom transfer radical polymerisation

BODIPY	Boron-dipyrromethene
DASA	Donor–acceptor Stenhouse adducts
D_h	Hydrodynamic diameter
DLS	Dynamic light scattering
$\bar{D}_M$	Molar-mass dispersity
DOX	Doxorubicin
DP	Degree of polymerisation
DPD	Dissipative particle dynamics
E_a	Activation energy
eq.	Equivalents
EVE	Ethyl vinyl ether
FRET	Förster resonance energy transfer
g	Gram(s)
G3	Third generation Grubbs' catalyst
h	Hour(s)
HPTS	8-Hydroxypyrene-1,3,6-trisulfonic acid
HRMS	High-resolution mass spectrometry

HRP	Horseradish peroxidase
Hz	Hertz
J	Coupling constant
K	Kelvin
kDa	Kilodalton(s)
kV	Kilovolt(s)
λ_{Em}	Emission wavelength
λ_{Ex}	Excitation wavelength
Liposome	Self-assembled structure formed of lipid molecules
$\text{LogP}_{\text{oct/SA}}$	Surface area-normalized octanol/water partition coefficients
L_{TEM}	Number-average length determined by TEM
MHz	Megahertz
min	Minute(s)
mL	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
mmol	Millimole(s)

M_n	Number average molecular weight
mol	Mole(s)
ms	Millisecond(s)
mV	Millivolt(s)
mW	Milliwatt(s)
M_w	Weight average molecular weight
NB-Amine	Tertiary amine substituted norbornene monomer
NB-COOH	Carboxylic acid substituted norbornene monomer
NB-MEG	Ethylene glycol monomethyl ether substituted norbornene monomer
NB-NI	Naphthalimide substituted norbornene monomer
NB-PEG	Polyethylene glycol substituted norbornene monomer
NB-Pyr	Pyrene substituted norbornene monomer
nm	Nanometre(s)
NMP	Nitroxide-mediated radical polymerisation
NPs	Nanoparticles
OD	Optical Density
p	Packing parameter

PB	Phosphate buffer
PD	Dispersity as determined by DLS
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PISA	Polymerisation induced self-assembly
PMMA	Poly(methyl methacrylate)
Polymersomes	Self-assembled structure formed of polymers
ppm	Parts per million
PS	poly(styrene)
RAFT	reversible addition-fragmentation chain-transfer
RDRP	reversible deactivation radical polymerisations
R_g	Radius of gyration
R_h	Hydrodynamic radius
RI	Refractive index
ROMP	Ring-opening metathesis polymerisation
ROMPISA	Ring-opening metathesis polymerisation induced self-assembly
rpm	Revolutions per minute

SAXS	Small-angle X-ray scattering
SEC	Size-exclusion chromatography
SiRNA	Short interfering RNA
SLS	Static light scattering
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
T_g	Glass transition temperature
THF	Tetrahydrofuran
UA	Uranyl acetate
UV	Ultraviolet
UV-vis	Ultraviolet-visible
wt%	Weight percentage
$Z_{\text{avg,DLS}}$	Intensity weighted mean hydrodynamic size determined by DLS
δ	Chemical shift
ζ	Zeta potential

Declaration of Authorship

This thesis is submitted to the University of Birmingham in support of my application for the degree of Doctor of Philosophy. No material contained here has been submitted for any other degree, or at any other institution.

The work presented (including data generated and data analysis) was carried out by myself between October 2019 and October 2023 except in the case outlined below:

Chapter 2 - High resolution mass spectrometry of norbornene monomers was performed by Dr Christopher Williams at the University of Birmingham.

Chapter 3 - High resolution mass spectrometry of norbornene monomers was performed by Dr Christopher Williams at the University of Birmingham.

Chapter 4 – Small angle X-ray scattering analysis and modelling was performed by Dr Matthew Derry at Aston University.

Chapter 1. Introduction

1.1 Understanding the biological world

1.1.1 The role of compartmentalisation in biology

Our bodies are compartmentalised into systems of organs, which are each responsible for managing a different process of homeostasis. Organs are made up of tissues which are groups of cells with similar functions. Cells are the basic functional and structural unit of a living organism and, at the simplest level, compartments. These cells are then further subdivided into other compartments, known as organelles (Figure 1.1).

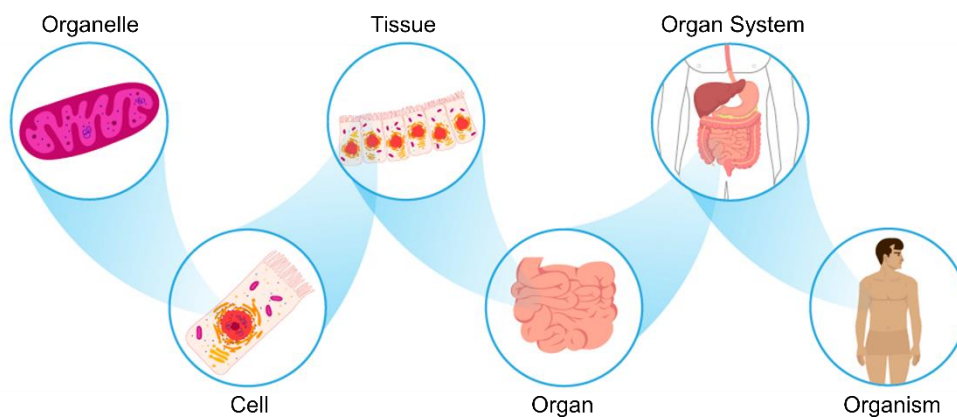


Figure 1.1. Schematic illustration of the levels of organisation in multicellular organisms. Adapted from ref.¹

Compartmentalisation of molecules into distinct environments by semipermeable structures is the key to structural organisation in nature.² These compartments are formed from amphiphilic lipids (most commonly phospholipids), comprised of a polar head group and non-polar tails (Figure 1.2). Due to the difference in hydrophobicity between the two sections, the phospholipid molecules spontaneously aggregate to desolvate their hydrophobic tails in the interior of the structure and minimise the free energy cost of forming ordered cage structures of surrounding water molecules.³ In doing so, phospholipids form two major morphologies: spherical micelles, with the tails inward, or bilayers, with the hydrophobic tails sandwiched

between the hydrophilic head groups. In addition, planar phospholipid bilayers are energetically unfavourable due to the interactions between water molecules and the exposed edges. As such, these structures spontaneously close to form a sealed compartment, termed vesicle or liposome (Figure 1.2).

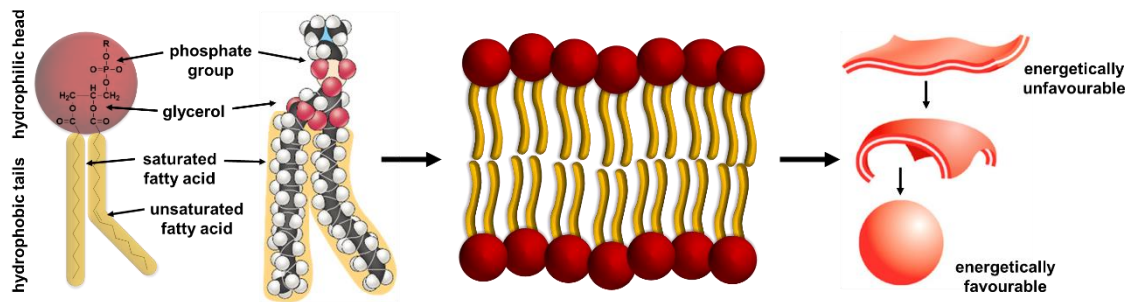


Figure 1.2. Chemical structure of a phospholipid molecule; structural organisation of phospholipids forming a planar bilayer; spontaneous closure of phospholipid bilayer to form a sealed compartment.

There are many benefits to compartmentalisation within living cells, for example processes can be regulated by the co-localisation of the reagents resulting in faster reaction rates due to high local concentrations.² Compartmentalisation can also provide a protective effect, excluding degradative molecules when accommodating sensitive entities (e.g. genetic material) and ensuring harsh reaction conditions (e.g. the low-pH environment found within endosomes) do not harm the rest of the cell.⁴ However, these benefits are conditional on the ability to regulate the transport of a broad range of materials into and out of these compartments by different transport mechanisms.

1.1.2 Material trafficking in biological structures

Over time, nature has developed the ability to regulate the passage of a range of substrates, from ions and small molecules to larger structures such as enzymes and proteins, across membranes with remarkable selectivity and specificity, facilitating the regulation of multiple

cellular processes.⁵⁻⁸ This is made possible through the cooperation of several membrane transport mechanisms, including diffusion, transport-mediated entry, peptide translocation domains, exocytosis, pinocytosis, and phagocytosis.⁹⁻¹⁴

1.1.2.1 Passive transport

Passive diffusion is the simplest mechanism available in the transport of material across the membrane (Figure 1.3). The molecule is dissolved within the membrane, diffuses across the membrane, and then dissolves into the aqueous solution on the other side. The rate and direction of material transfer is primarily dictated by the concentration gradient of the molecule across the membrane. In passive diffusion, molecules must have some solubility in the hydrophobic domain of membranes. It therefore occurs at a sufficient rate for small, relatively hydrophobic molecules, such as gases (e.g. oxygen and carbon dioxide), steroids and ethanol.⁶

Molecules that are more hydrophilic, charged, or larger in size cannot permeate the hydrophobic phospholipid membrane as easily, resulting in a slow rate of passive diffusion. Instead, these molecules are transported by facilitated diffusion, also known as carrier-mediated or passive-mediated transport (Figure 1.3). Similar to passive diffusion, the process is driven by electrochemical gradients across the membrane and so is a thermodynamically favourable process that does not require an external energy source.

However, to facilitate the passage of the hydrophilic molecules across the hydrophobic membrane, channel proteins (sometimes called transmembrane integral proteins) are embedded in the membrane (Figure 1.3). These provide a hydrophilic passageway to the transporting molecules, removing the requirement for the transporting molecule to dissolve

in the hydrophobic membrane. Formed from a specific arrangement of amino acids, channel proteins may either be non-gated, such as aquaporin, which remains open all the time to allow the free passage of water to flow through the membrane. In contrast, gated channel proteins, e.g. potassium channels, remain closed until a chemical or electrical signal is sensed.¹⁵ The ability to gate an ion channel allows electrical energy to be built up inside the cell, a key requirement in nerve function.¹⁶ The regulated opening and closing of ion channels thus provides cells with a sensitive and versatile mechanism for responding to a variety of environmental stimuli, allowing the movement of material across membranes to be coordinated with other processes. Molecules such as charged ions (e.g. Na^+ , Cl^- , K^+) and water are transported across the membrane in this manner.¹⁷

Some cellular metabolites, such as glucose, are unable to pass through the narrow passageway created by a channel protein. Here, carrier proteins bind to the glucose molecules and transport the molecule across the membrane before release on the other side (Figure 1.3). Due to the requirement for binding, carrier proteins are more selective to the molecules that they transport than most channel proteins. This method of facilitated diffusion is typical for molecules such as sugars, amino acids, and nucleosides.¹⁰

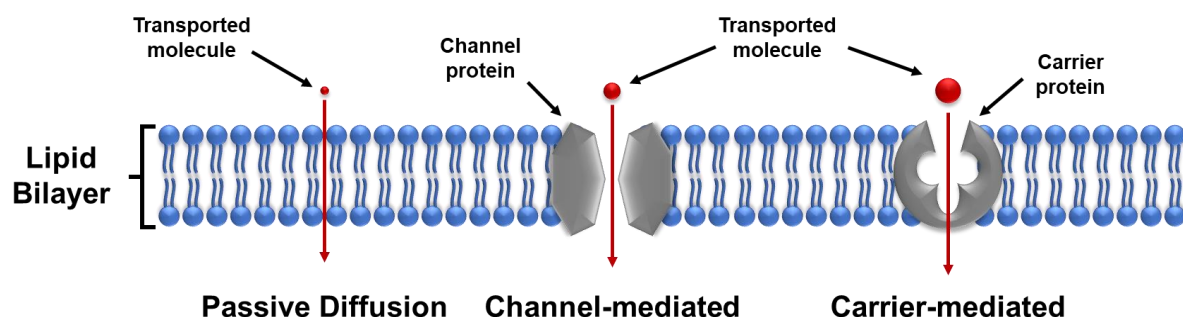


Figure 1.3. Mechanisms of passive membrane transport across the phospholipid bilayer.

1.1.2.2 Active transport

The direction of transport for molecules crossing the membrane by passive diffusion is determined by concentrations across the membrane. However, in many cases, biology must transport molecules against their concentration gradient. For example, to remove waste products, accumulate nutrients (that are at a low concentration in surrounding environment) and to precisely move material from one compartment to another. During this process, known as active transport, material is moved in the energetically unfavourable direction, either by input of an external energy source (e.g. adenosine triphosphate (ATP)) or driven by a difference in electrochemical potential when ions are transported across the membrane.

This process uses carrier proteins which maintain the high levels of specificity required and are involved in the transport of a range of molecules, including sugars, most amino acids, organic acids and a number of inorganic ions such as sulphate, phosphate and potassium.¹⁸

1.1.2.3 Bulk transport

Although there are a range of mechanisms for the transport of molecules through a membrane, there are several types of material that are too large to pass through the phospholipid bilayer, including proteins, bacteria, and organelles. To transport these vital materials around, nature must utilise a mechanism known as bulk transport. These mechanisms involve enclosing the substances to be transported in their own small membrane-bound compartment, which can then bud from or fuse with the cell membrane to move the substance across.

Depending on the direction of the transport of material, bulk transport is separated into two categories: endocytosis and exocytosis (Figure 1.4). Endocytosis is the process of moving

material into a cell, whereas exocytosis is the reverse process, with material moving from the inside to the outside of a cell. Both of these mechanisms are necessary for crucial functions in biology, such as taking in nutrients for cellular growth, function and repair; capturing pathogens or other unknown substances that may endanger the organism; and disposing of old or damaged cells.¹⁹

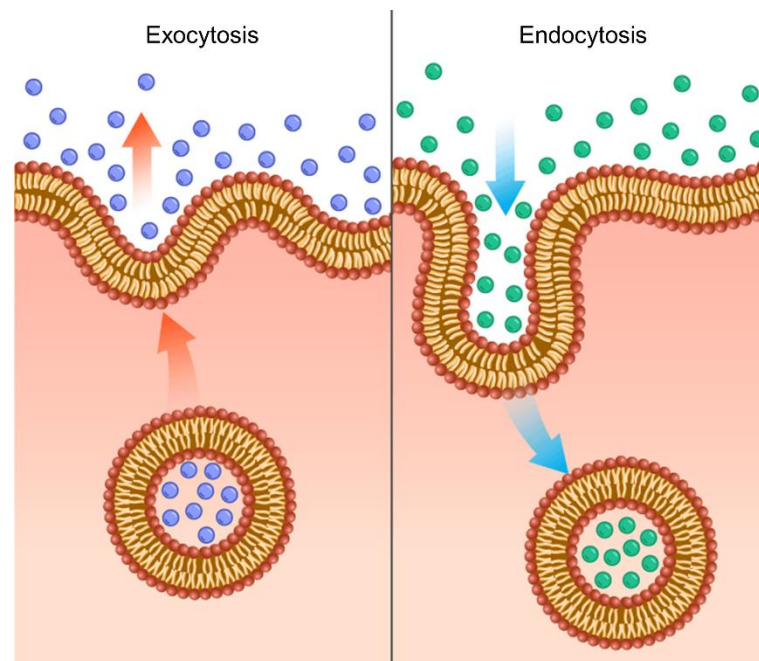


Figure 1.4. Bulk membrane transport across the membrane – exocytosis and endocytosis. Adapted from ref.²⁰

Both endocytosis and exocytosis involve the fusion of phospholipid membranes, merging or separating compartments, to transfer material from one side of the cell membrane to another. This process is very well controlled because the fusion of phospholipid membranes is not normally spontaneous. It carries a large energetic barrier due to the need to increase phospholipid membrane curvature/tension and overcome charge repulsion from approaching phospholipid headgroups.²¹ The energy required to overcome this barrier is obtained from

additional biological machinery, called soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs).²²

SNAREs are a family of membrane-embedding proteins that, when situated on two phospholipid membranes, mediate the fusion of the phospholipid bilayers over a two-step process.²³ Firstly, as the proteins are brought into close proximity they form complementary interactions with one another, leading to their spontaneous self-assembly and tethering the two together in a metastable configuration. Then, often following an external stimulus (e.g. Ca^{2+} trigger), this intermediate collapses and promotes complete phospholipid membrane fusion (Figure 1.5).²⁴

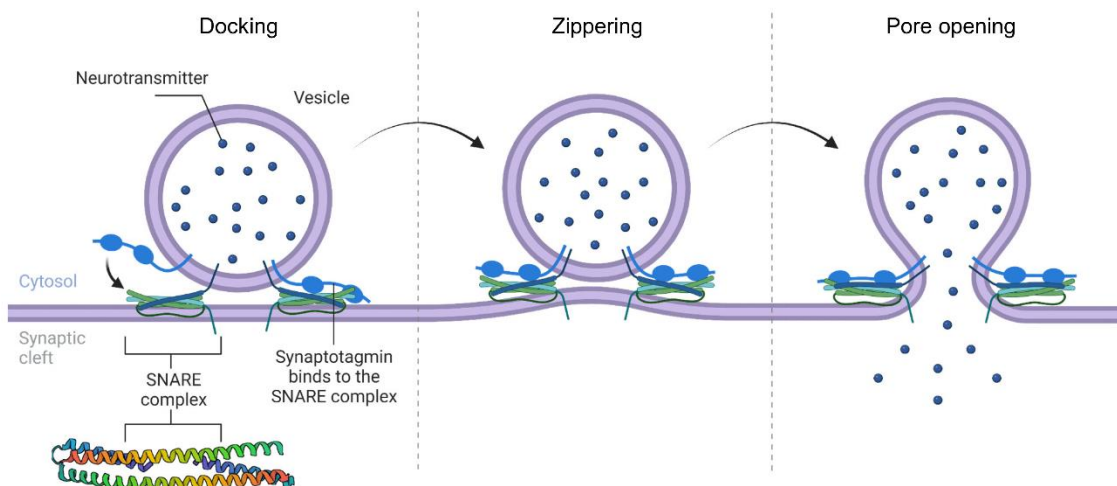


Figure 1.5. Mechanisms of SNARE proteins in membrane fusion. Adapted from Azimi *et al.*²⁵

In addition to this, bulk transport also utilises molecular motors, such as myosins, kinesin and cytoplasmic dynein, called motor proteins.²⁶ These proteins use the energy derived from repeated cycles of ATP hydrolysis to drive cargo transport along the filamentous meshwork that comprises the cytoskeleton.²⁷

1.1.2.4 Biomimetic strategies for material transport

Inspired by the intricate material trafficking mechanisms in biological systems, researchers have strived to develop biomimetic strategies to replicate and harness these processes for various applications. Biomimetic approaches aim to mimic the principles of intracellular transport to improve drug delivery, create self-assembling nanosystems, and engineer functional materials.

Examples include the development of synthetic vesicles or liposomes that mimic the structure and behaviour of biological vesicles for applications such as cascade nanoreactors, synthetic cells and nanosensors.^{2, 28, 29} Similarly, molecular motor-driven systems have been engineered to transport cargo along synthetic microtubules or actin filaments, enabling controlled movement and delivery of materials.³⁰⁻³³ Continued advancement in this area, focussing specifically on selectivity, efficiency, and autonomy, would result in the generation of highly complex synthetic systems with the ability to perform incompatible reactions at the same time in different nanoscale containers.

1.2 Polymers

Polymers are long chained molecules composed of a series of smaller units, termed monomers. The monomers can either be linked covalently or can be bound by non-covalent intermolecular interactions to form long chains or networks. This connectivity between the units is what gives the polymer the properties that differ from a group of individual monomer units.

There are two main types of polymers: natural biopolymers, such as polypeptides, polysaccharides and nucleic acids, and synthetic polymers, for example plastics, rubbers and

fibres. Due to their versatile properties and inexpensive, scalable synthesis, polymers are ubiquitous with modern day living and are used in almost every industry, including energy storage, transport, textiles, medical, engineering and electronics.^{34, 35}

1.2.1 Properties of polymers

Existing polymer synthesis methods rarely generate polymers of a precise number of monomer units. Therefore, a single polymer sample will usually contain a range of chain lengths and molecular weights. The amount of variation in chain lengths can affect the bulk properties of the polymer, therefore it is important to quantify this distribution.

A polymer's molecular weight can be described in two ways: number average molecular weight (M_n), and weight average molecular weight (M_w). M_n is the averaged molecular mass with equal contribution of all the chains regardless of their mass (Equation 1.1). M_n can be used to determine the average number of monomer units per polymer chain, also termed the mean degree of polymerisation (DP).

$$M_n = \frac{\sum n_i M_i}{\sum n_i} \quad 1.1$$

where n_i equals the number of moles of each chain with molecular weight M_i .

By comparison, M_w is more representative of higher weight chains which account for a greater proportion of the total molecular mass (Equation 1.2).

$$M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \quad 1.2$$

The dispersity (D_M) of a polymer sample is described as the ratio between M_n and the M_w (Equation 1.3). A polymer is described as monodisperse if all chain lengths are the same (D_M

= 1). However, this is a rare occurrence for synthetic polymers, with a sample typically containing chains of different lengths, described as polydisperse. The greater value of $\bar{D}_M$, the greater variation in chain length.

$$\bar{D}_M = \frac{M_w}{M_n} \quad 1.3$$

Determination of M_n and M_w is typically achieved using size-exclusion chromatography (SEC), a technique which separates molecules in a solution based on their size by filtration through a gel column. Unlike other techniques (e.g. affinity chromatography), the molecules do not bind to the chromatographic column. Instead, the column is packed with porous beads of specific range of pore size which can be used to determine the size of the molecules.

Once the sample has entered the column, molecules that are larger than the pore size are unable to diffuse into the beads, meaning they have a shorter path length and so pass through the column quickly. By comparison, molecules that are smaller can penetrate the pores to varying degrees, increasing their path length, resulting in longer retention times within the column (Figure 1.6). From this retention time, M_n and M_w can be estimated by comparison with a reference sample of known molecular weight, typically poly(methyl methacrylate) (PMMA) or poly(styrene) (PS).

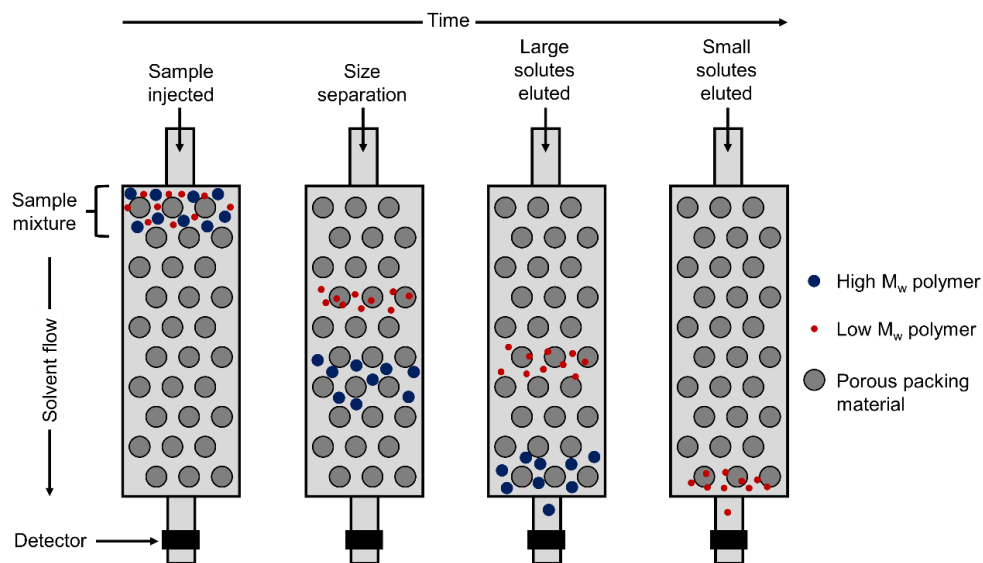


Figure 1.6. Schematic diagram of the separation of two distinct polymers with different molecular weights by SEC.

Multiple methods can be used to detect the eluting polymer from the column, such as changes in refractive index (RI), or ultraviolet (UV) spectroscopy. As the molecular weight of the analysed sample is calculated against a calibration, which is normally not the same type of polymer, the value is usually an approximation.

The molecular weight of a polymer influences its physiochemical properties, which can be tuned by altering a polymer's average chain length, without changing the chemistry. A high molar mass results in properties such as increased viscosity, good chemical resistance, and high mechanical strength. However, the bulk properties of a polymer, including glass transition temperature (T_g), solubility and optical properties, can be controlled by topology, composition and functionality (Figure 1.7).

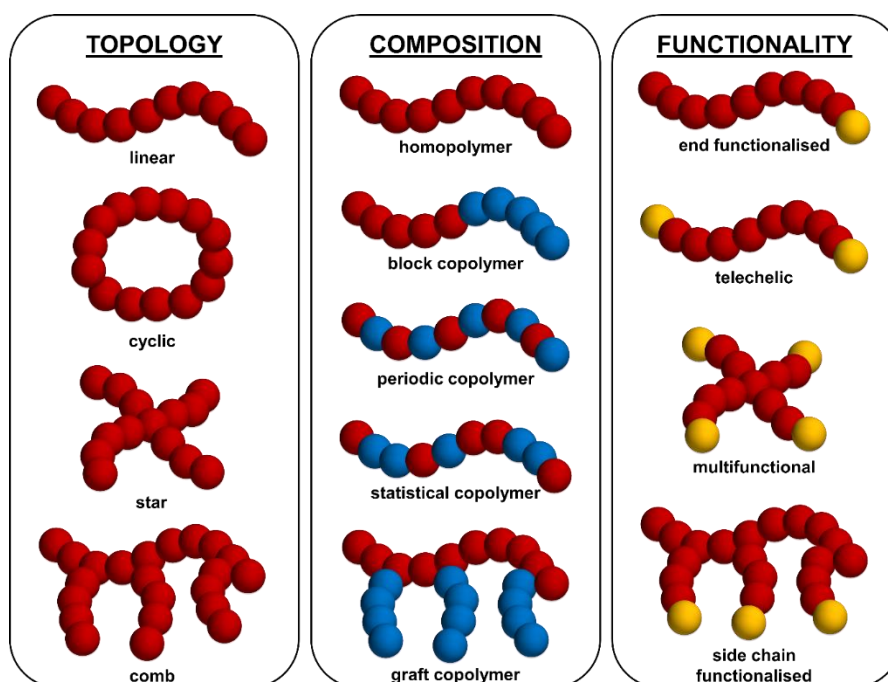


Figure 1.7. Different topologies, compositions, and functionalities of polymers.

For example, polymers formed from siloxane, such as polydimethylsiloxane (PDMS), are typically oils or rubber-like substances with a low T_g value due to the flexibility in the Si-O backbone. As well as the bonds that form the backbone of the polymer themselves, the functional groups that are located along the polymer chain can also impact the overall polymer properties. Poly-paraphenylene terephthalamide, more commonly known as Kevlar, has a backbone that consists of amide groups that form inter-molecular hydrogen bonds between different polymers chains. Because of this, Kevlar has a tensile strength of over 3000 MPa and can withstand temperatures up to 260 °C.³⁶

A polymer whose monomers are all identical is termed a homopolymer. However, two or more different monomers can be combined to form a copolymer. The properties of the copolymer depend on the monomers used, as well as the configuration of the monomers. This configuration can be categorised as: alternating, random, block, periodic or graft.

1.2.2 Polymerisation methods

Synthetic polymer synthesis can generally be categorised into two types: chain-growth and step-growth polymerisation. Each category uses monomers with distinct characteristics and display different growth patterns (Figure 1.8).

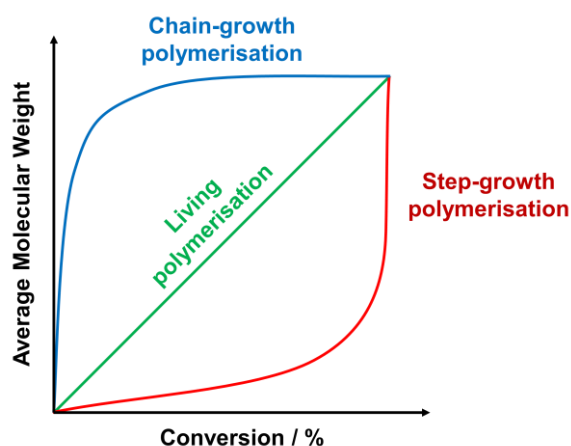


Figure 1.8. Increase in polymer molecular weight versus monomer conversion for chain-growth, step-growth, and living polymerisation.

Step-growth polymerisation proceeds stepwise by intermolecular reaction of polyfunctional reactants, often with loss of a small molecules (e.g. water). During step-growth polymerisation, all polymer chains are growing at the same time and so the length of oligomers remains relatively small at the start of the reaction. Later in the polymerisation, when much of the monomer has reacted, combination of the growing oligomers occurs which in turn leads to a rapid increase of molecular weight at high conversion (Figure 1.8).

There are three stages within chain-growth polymerisation: (1) initiation, in which a reactive species (i.e. radical, cation or anion) is formed from which the polymer chain can grow; (2) propagation, subsequent addition of monomers to the growing chains increases the chain length whilst maintaining the reactive centre; and (3) termination, where the reactive species

is irreversibly removed and the chain can no longer propagate. The reactive species grows quickly during propagation, leading to the formation of long polymers in a short period of time (Figure 1.8).

1.2.2.1 Living polymerisation

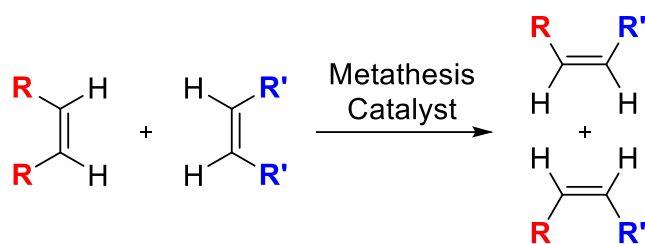
If the ability of a growing polymer chain to terminate has been removed, then the polymerisation is classified as “living” (Figure 1.8).³⁷ In this case, the polymer chains continue to propagate until all the monomer is consumed and, even after full conversion, the polymer chains remain active. This allows for the addition of a secondary monomer supply allowing the formation of block copolymers. Some anionic chain polymerisations proceed as living polymerisations under conditions where no viable chain terminating reactions occur. However, for other forms of chain-growth polymerisation, the complete removal of termination steps cannot be achieved.³⁸

To overcome this, radical or cationic chain-growth polymerisations can be endowed with “living character” by prolonging the lifetime of the reactive species through the introduction of dormant states. Several polymerisation methods have been developed to achieve this, most notably reversible deactivation radical polymerisations (RDRP), such as atom transfer radical polymerisation (ATRP), nitroxide-mediated radical polymerisation (NMP) and reversible addition-fragmentation chain-transfer (RAFT) polymerisation.

As well as the absence of chain termination, living polymerisation is often characterised by a faster rate of initiation than propagation, ensuring all polymer chains grow at the same time and rate.³⁸ As a result, polymers obtained *via* living polymerisation techniques have controlled lengths, narrow dispersity and control over end-group functionality.

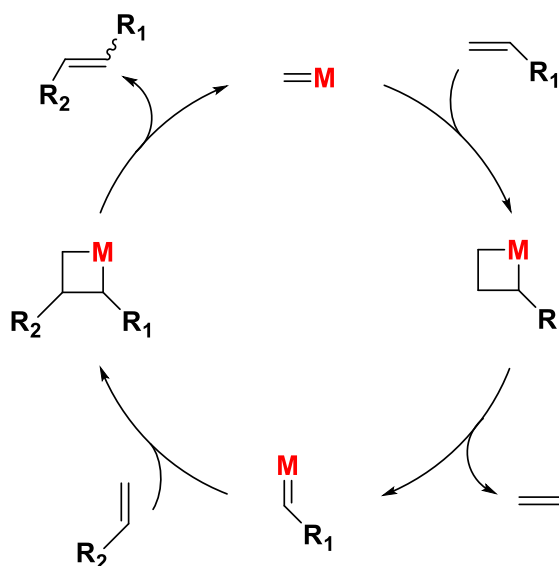
1.2.2.2 Ring-opening metathesis polymerisation

In alkene metathesis, substituents are exchanged between two alkenes by the scission and regeneration of the carbon-carbon (C-C) double bonds (Figure 1.1). The process is facilitated by a transition metal catalyst with a metal-carbon double bond.³⁹ A huge range of catalysts have been developed over the years, utilising various transition metals.⁴⁰



Scheme 1.1. Generic reaction scheme for alkene metathesis.

The mechanism of alkene metathesis starts with the [2+2] cycloaddition between the alkene and the metal-carbon double bond, forming a metallacyclobutane intermediate (Scheme 1.2).⁴¹ This metallacycle undergoes cycloelimination to produce a new alkene and alkylidene.⁴² Alkene metathesis is utilised in a variety of reactions, including cross metathesis, ring-closing metathesis, ring-opening metathesis polymerisation, enyne metathesis and acyclic diene metathesis polymerisation.



Scheme 1.2. Hérison and Chauvin mechanism for alkene metathesis, showing the formation of the four-membered metallacycle.

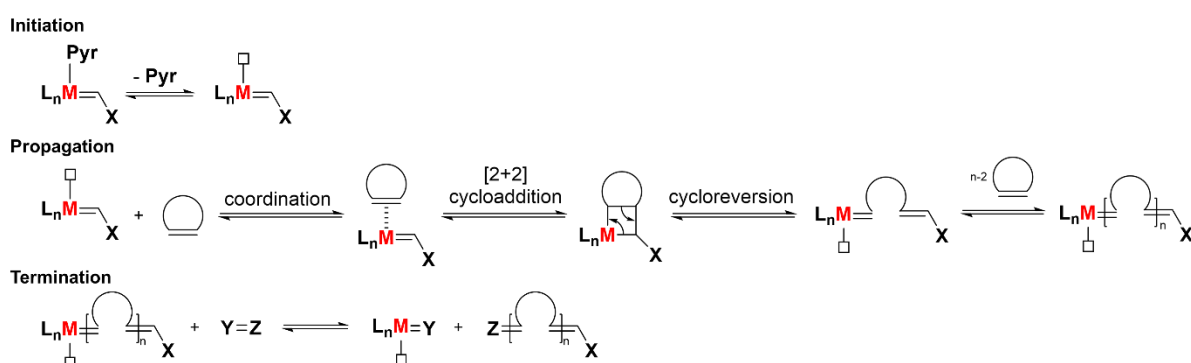
Ring-opening metathesis polymerisation (ROMP) is a form of chain-growth polymerisation that utilises the mechanism of alkene metathesis. Monomers used in ROMP are cyclic alkenes, such as cyclobutene, cyclopentene, cis-cyclooctene and norbornene, all of which share relatively high ring strain. During polymerisation, the strain is released as the ring is opened and provides an enthalpic driving force for the reaction. However, the entropy of the system decreases as polymerisation proceeds due to a loss in translational degrees of freedom of the growing polymer chain. As such, the enthalpic contribution of ring strain must supersede the entropic cost for polymerisation to occur.⁴³

Although initially employing undefined catalytic systems, modern day ROMP utilises well-defined transition metal complexes, many of which are commercially available (Schrock, Grubbs, Hoveyda catalysts).⁴⁴

In the mechanism of ROMP, the reaction is initiated by the formation of an open coordination site at the metal catalyst centre *via* loss of a ligand, typically pyridine (Scheme 1.3). This allows

coordination of the cycloalkene to the metal and the formation of a metallacyclobutane intermediate. This intermediate can then undergo cycloreversion to form a metal alkylidene complex and a new alkene, completing the first propagation cycle.

Following this, the metal centre can further react with a second cycloalkene to form a new metallacycle. This process is continually repeated until the monomer source is fully consumed, or the metal catalyst is cleaved from the polymer *via* a termination step, such as cross metathesis. Any unsaturation associated with the monomer is conserved as it is converted to polymer, unlike typical alkene addition polymerisations such as acyclic diene metathesis.⁴⁵



Scheme 1.3. Mechanism of ring-opening metathesis polymerisation.

Transition metal-catalysed ROMP proceeds homogeneously with clearly defined active species. As such, modification of the catalytic activity is possible by ligand design and provides a route to control polymer molecular weight and dispersity. Of particular interest are catalysts with a ruthenium metal centre. Compared to other transition metals, Ru-based catalysts, also known as Grubbs' catalysts, offer broad functional group tolerance, as well as stability to air and water.⁴⁶ Since their initial discovery in 1995 for use in norbornene polymerisation, Grubbs' catalysts have undergone several iterations to further improve both their activity and stability (Figure 1.9).⁴⁷

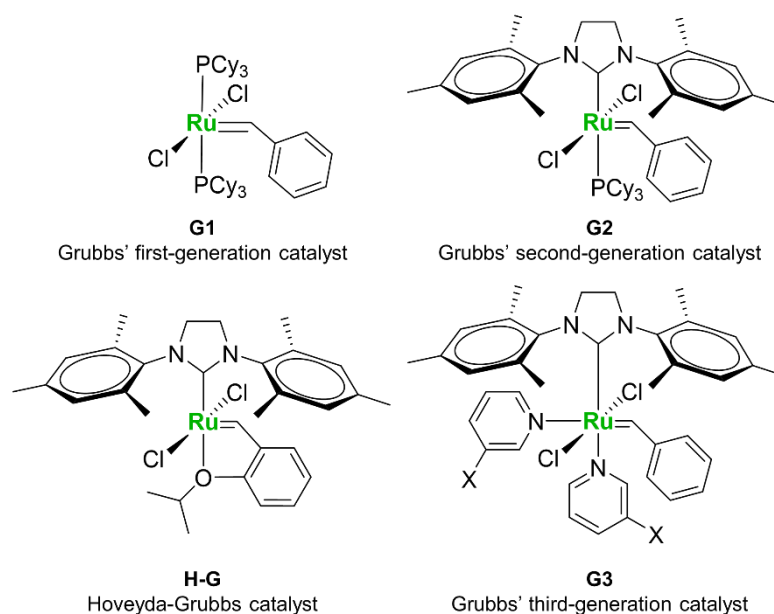


Figure 1.9. Chemical structures of different generations of Grubbs' metathesis catalysts.

The generation of highly active and stable catalysts has led to the development of living ROMP that can produce block copolymers of varying compositions with good molecular weight control and narrow dispersity.⁴⁸⁻⁵⁰

1.3 Amphiphilic block copolymer self-assembly in solution

Amphiphilic block copolymers contain two discrete blocks that are chemically linked: one is solvophilic and the other is solvophobic. In solution, similarly to lipids or surfactants, amphiphilic block copolymers will spontaneously self-assemble (Figure 1.10). This is driven by the reduction in unfavourable interactions between the solvophobic block and the solvent, known as the solvophobic effect.⁵¹ The resulting self-assembled structure is influenced by a number of factors, such as copolymer structure, polymer composition (including molecular weight and dispersity), core solvophobicity, thermal properties of the polymers (including crystallinity, T_g and rigidity), solvent composition and polymer-polymer interactions (e.g. Hydrogen bonding, π - π stacking).

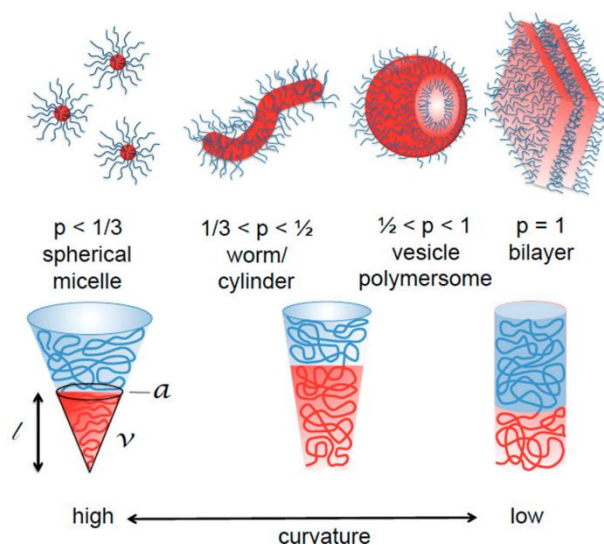


Figure 1.10. Amphiphilic block copolymer morphologies in solution. Adapted from Nunes *et al.*⁵²

The morphology of the self-assembly can, in part, be predicted by calculation of the packing parameter, p , (Equation 1.4).⁵³ Here, v is the volume of the solvophobic block, a_0 is the optimal contact between the solvophilic block and the solvent, and l_c is the length of the solvophobic block (Figure 1.10). In most cases, block copolymer self-assemblies have fast unimer exchange dynamics, meaning their assemblies are under thermodynamic control and results in the formation of the assembly lowest in energy. However, if unimer rearrangement and exchange is slow compared to the rate of assembly then out-of-equilibrium, “kinetically trapped” structures can be formed. When this occurs, p is no longer a good predictor for morphology.

$$p = \frac{v}{a_0 l_c} \quad 1.4$$

The self-assembly of amphiphilic block copolymers in solution is well established, with a range of nano-object morphologies, including spheres, worms, vesicles, lamellae, disks and toroids having been reported.⁵³⁻⁵⁵ Traditionally, block copolymers have been prepared by laborious post-polymerisation methods, such as solvent switching, film rehydration, bulk hydration,

electroformation, and nanoprecipitation.^{56, 57} However, these techniques require dilute solutions and multi-step syntheses (requiring the isolation of the polymer prior to self-assembly), limiting their potential economical scale-up to an industrial synthetic process.

1.3.1 Polymerisation induced self-assembly

Typically, diblock copolymers are synthesised in a good solvent for all blocks so they are freely soluble. Instead, in polymerisation induced self-assembly (PISA) a solvophilic precursor polymer is chain-extended using a solvophobic block monomer, for which the homopolymer is insoluble in the reaction solvent. As the second polymer block lengthens it becomes insoluble and, after a critical DP is reached, the newly formed block copolymers spontaneously self-assemble (Figure 1.11).⁵⁸

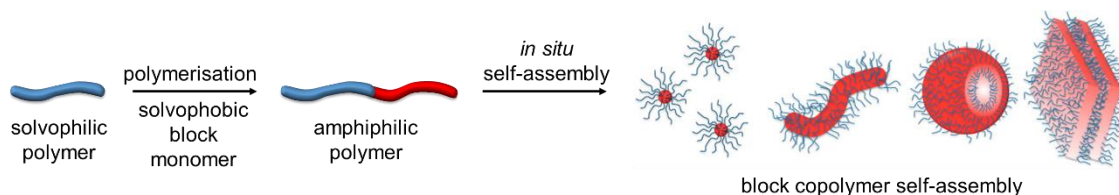


Figure 1.11. Schematic representation of polymerisation induced self-assembly (PISA). Adapted from Nunes *et al.*⁵²

This process can be completed in a single synthetic step at high solids concentration, with both polymerisation and self-assembly occurring simultaneously. Moreover, PISA has relatively short reaction times due to unreacted monomer being preferentially located within the growing nanoparticle, increasing the rate of reaction due to high local concentration.

PISA has been utilised in a wide array of solvents, including water, polar, non-polar, ionic fluids and supercritical CO₂.⁵⁹⁻⁶³ A range of morphologies have been reported e.g. spheres, worms,

vesicles, rods and lamellae, which can be tuned by careful manipulation of the relative volume fractions of the different blocks.⁶⁴

Due to the requirement for discrete polymer blocks, a “living” polymerisation technique is needed for PISA to be successful. As such, reversible-deactivation radical polymerisation methods (e.g. ATRP, RAFT) are typically utilised in PISA.⁶⁵

1.3.2 Ring-opening metathesis polymerisation induced self-assembly

Recently, ring-opening metathesis polymerisation induced self-assembly (ROMPISA) has been explored as an alternative synthetic methodology towards self-assembled nanostructures. The rapid polymerisation kinetics achievable with modern day Ru-based catalysts and backbone rigidity of the resulting polymer allows access to kinetically trapped, out-of-equilibrium morphologies – a key advantage over other PISA methodologies.

Research into ROMPISA has been particularly focussed on the synthesis of polynorbornene-based nano-objects.⁶⁶ The radical-free nature of the polymerisation and functional group tolerance of Ru-based catalysts allows access to a wide range of chemistries under mild reaction conditions.⁶⁷ As such, ROMPISA allows for the facile synthesis of functional polynorbornene block copolymers with excellent molecular weight control and narrow dispersity.

ROMPISA was first reported in the preparation of block copolymer nano-objects by Xie and co-workers.⁶⁸ In this report, spherical micelles, with diameters between 100 to 220 nm, were prepared when a poly(2,3-bis(2-bromoisobutyryloxymethyl)5-norbornene) (PBNBE) macroinitiator was chain-extended in dry toluene using an oxanorbornene dicarboxylic acid

dimethyl ester (ONBDM) monomer and first generation Grubbs' catalyst, $(\text{PCy}_3)_2(\text{Cl})_2\text{Ru}=\text{CHPh}$, under nitrogen (Figure 1.12).

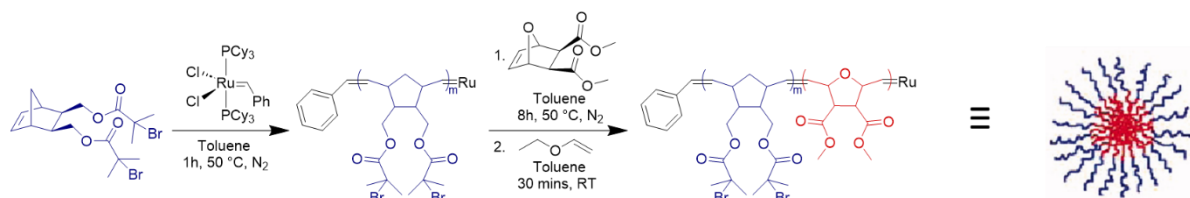


Figure 1.12. One-pot synthesis of shell-functionalised polymeric nanoparticle by ROMPISA. Adapted from Zhang *et al.*⁶⁸

Further progress in the development of ROMPISA was reported by Choi and co-workers, where polynorbornene-*b*-polyacetylene block copolymers (PNB-*b*-PA) were synthesised using a third generation Grubbs' catalyst in a range of organic solvents.⁶⁹ By varying the relative block lengths, morphologies from spherical micelles to long “nanocaterpillars” was observed (Figure 1.13). The researchers attribute the interesting morphology to the π - π interactions between the long PA segments and noted possible applications in nanoelectronics.

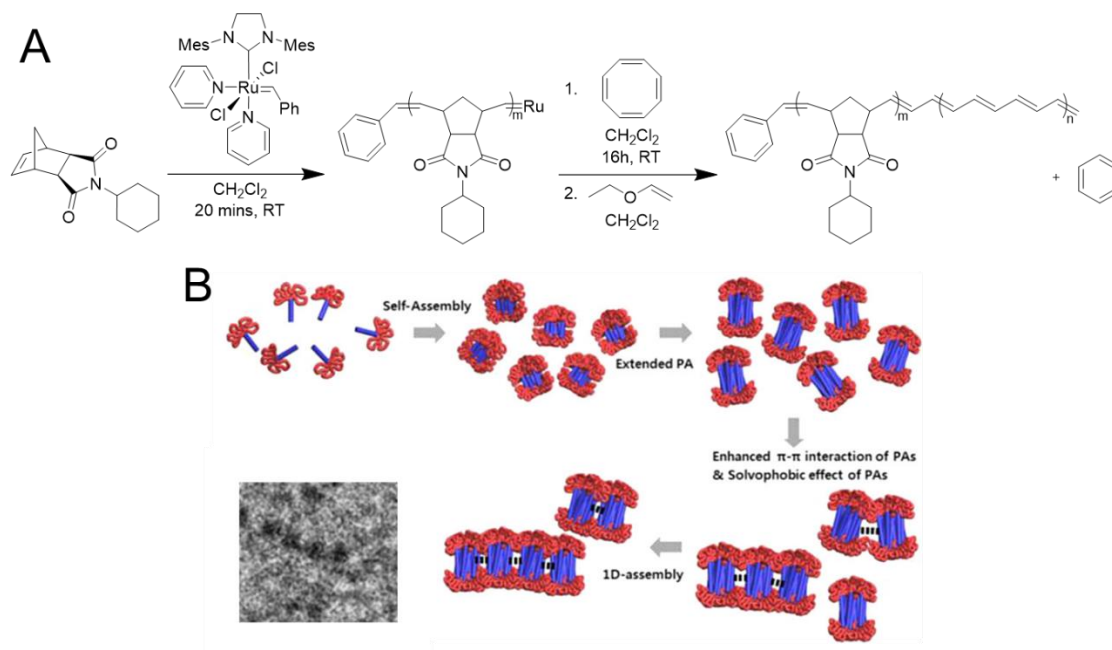


Figure 1.13. (A) Reaction scheme for the preparation of PNB-*b*-PA nano-objects by ROMPISA (B) Proposed mechanism for the *in situ* formation of PNB-*b*-PA into nanocaterpillars and transmission electron microscopy image of the final morphology. Adapted from Yoon *et al.*⁶⁹

1.3.2.1 Aqueous ring-opening metathesis polymerisation induced self-assembly

Following seminal work on the ring-opening metathesis polymerisation of norbornene in organic solvent,⁷⁰ progress has also been made to implementing ROMPISA in aqueous systems. Although Schrock catalysts require water and oxygen to be rigorously excluded, Ru-based catalysts are known to be far more tolerant to these conditions, permitting alkene metathesis in aqueous conditions.⁷¹ Research in aqueous ROMPISA is motivated by the fact that an improved capability to conduct ROMP in water would facilitate applications of polymerisation in biological media and in the preparation of nano-objects *via* aqueous dispersion polymerisation.

Gianneschi and coworkers have developed an aqueous ROMPISA methodology utilising a water-soluble ruthenium initiator (AquaMet) to produce enzyme-responsive polymer

nanoparticles (Figure 1.14).⁷² They first prepared a norbornene-functionalised dicarboximide peptide substrate that was then chain-extended using a quaternary amine-based phenyl norbornene dicarboximide. By varying the relative block lengths, morphologies from spherical micelles to framboidal vesicles were formed. The peptide sequence within the nano-objects was shown to cleave in the presence of the proteolytic enzyme thermolysin and result in a phase transition to large aggregates. This method demonstrates a route to enzyme-responsive materials that exhibit application in tissue targeting drug delivery at high weight percentage solids.

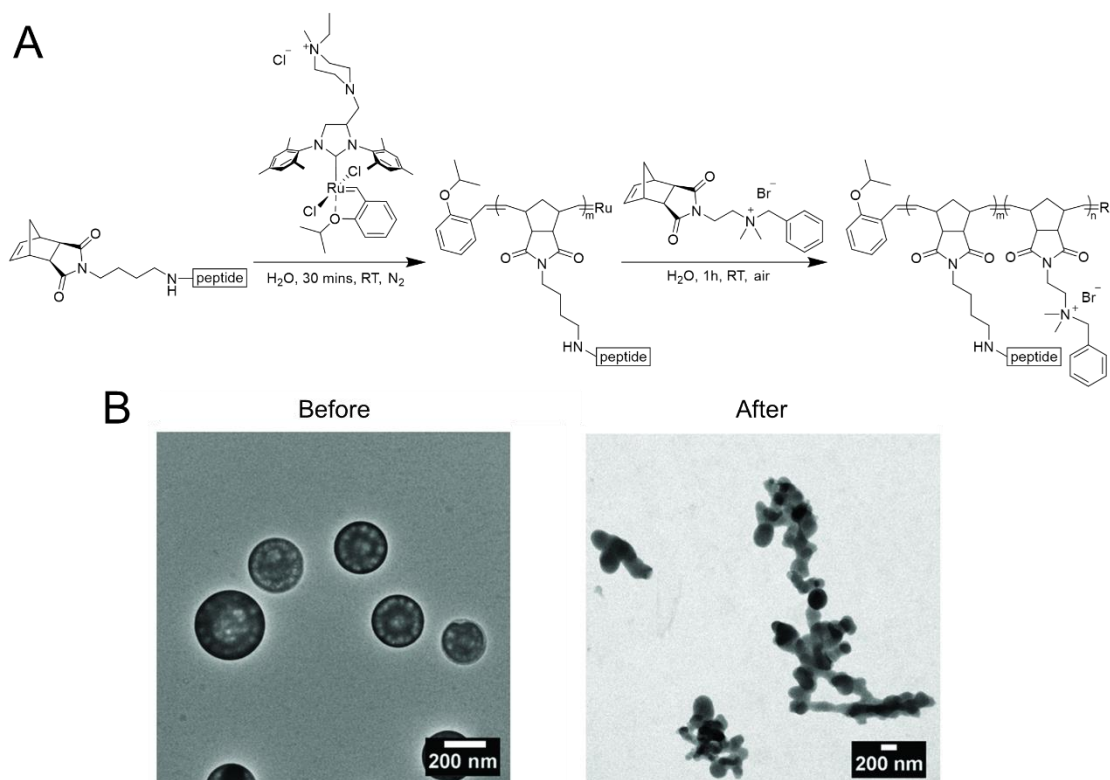


Figure 1.14. (A) Reaction scheme for the formation of peptide-functionalised diblock copolymers using AquaMet initiator. (B) Transmission electron microscopy images for peptide-functionalised nano-objects before and after incubation with thermolysin. Adapted from Wright *et al.*⁷²

Although water-soluble catalysts have been shown to achieve ROMPISA they still display poor catalytic activity compared to third generation Grubbs' catalyst (**G3**) in organic solvent and typically require additional additives e.g. Bronsted or Lewis acids to promote dissociation of phosphine or pyridine ligands.^{73, 74}

Hence, O'Reilly and co-workers have developed a methodology for conducting ROMPISA in aqueous conditions using a macroinitiator approach.⁷⁵ They first polymerised a tertiary amine-functionalised norbornene with a commercially available **G3** catalyst in tetrahydrofuran (THF), yielding a water-soluble macroinitiator. This was then transferred to an acidic phosphate buffer (PB2) (pH 2) solution and chain-extended with a core-forming monomer, forming nano-objects with various morphologies (Figure 1.15).

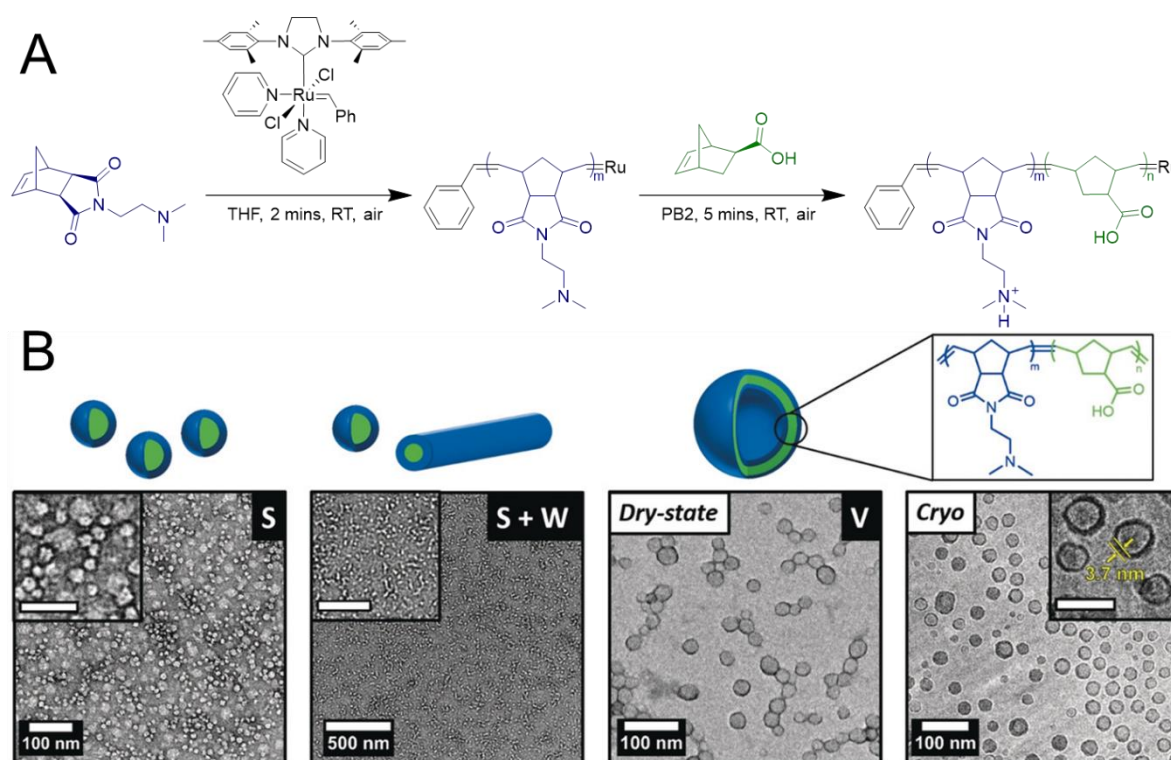


Figure 1.15. (A) Reaction scheme for the formation of nano-objects by aqueous ROMPISA using a macroinitiator approach. (B) Dry-state and cryo-transmission electron microscopy images of the different morphologies formed. Adapted from Foster *et al.*⁷⁵

The key advantage of this two-step process is the utilisation of very active catalytic species, with polymerisations reaching full conversion in under 90 seconds in mild reaction conditions. SEC analysis showed very good molecular weight control for all polymerisations, including when the solids concentration was increased to 20%. Nano-objects prepared in aqueous solution could find application in biological media (e.g. drug delivery).

This methodology for aqueous ROMPISA was further scoped in a subsequent report, in which computationally derived hydrophobicity measurements were obtained for a series of new monomers. These values were then used to predict the ability of each monomer to form nano-objects of various morphologies during aqueous ROMPISA.⁷⁶ As well as broadening the range of monomer functionalities available, the authors also identify trends between monomer architecture, such as anchor or terminal functional group, polymer hydrophobicity and nano-object morphology.

1.3.3 Characterisation techniques for polymer self-assemblies

Characterising the structure of polymer self-assemblies is vital to understanding the mechanisms that drive morphological transitions. However, many characterisation techniques have limitations when employed for self-assembled polymer structures and so it is important to utilise multiple methods to accurately determine physical properties, such as morphology, size, and dispersity. The most common methods utilised for particle characterisation are microscopy and scattering techniques.

1.3.3.1 Transmission electron microscopy

Transmission electron microscopy (TEM) is a microscopy technique that works under the same principle as a light microscope: radiation interacts with a sample to form an image of the

sample. Typically, TEM is carried out by depositing a sample in solution onto a thin carbon-coated copper mesh grid, followed by the removal of solvent. The grid is then irradiated with an electron beam under vacuum to which, when it reaches the sample, some of the electrons are transmitted, while the rest are scattered. The electrons that are transmitted through the sample are then measured and used to build the final image.⁷⁷

The removal of solvent is necessary for TEM, as the instrument operates under a vacuum to limit the interaction of the electron beam with gas molecules. As such, the particles are not being imaged in their native state in solution which can lead to considerable changes in particle morphology and size. Dehydration can lead to changes such as the collapse of the corona chains (that would normally be swollen in solvent), full particle collapse, or induce crystallisation, producing a different image to the structure of the particles in solution.⁷⁸

The amplitude and phase variations in the transmitted beam provide imaging contrast that is dependent on the number of scattered electrons, with heavier atoms scattering more electrons than lighter ones.⁷⁷ As such, to observe particles easily, the sample must scatter significantly more electrons than their support. However, due to the use of carbon TEM grids, the contrast is typically too low to sufficiently image carbon-based nanostructures.⁷⁹ To overcome the issue of low contrast, a heavy metal stain (e.g. osmium tetroxide, ruthenium tetroxide, uranyl acetate, ammonium molybdate or phosphotungstic acid) can be applied which will selectively bind to the grid (negative staining) or the particle (positive staining), enhancing the contrast difference between them (Figure 1.16).⁸⁰

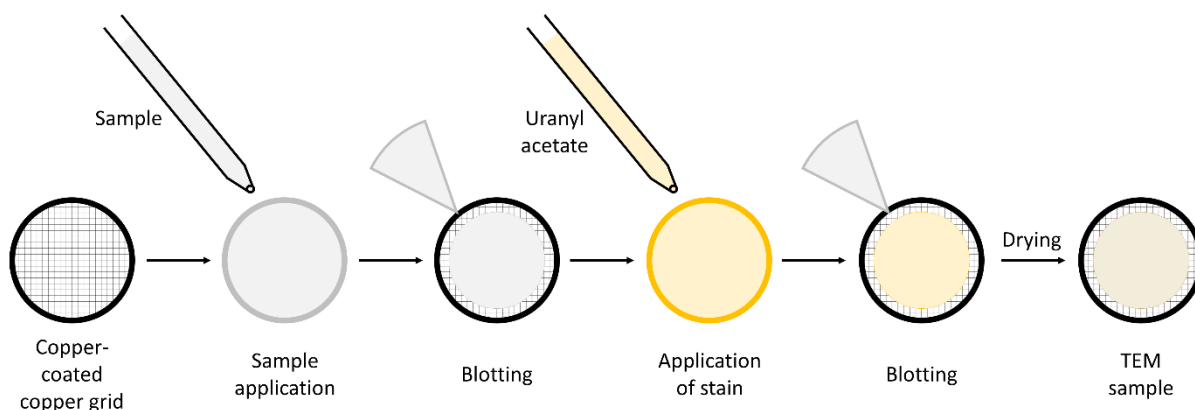


Figure 1.16. Schematic representation of the staining procedure in TEM sample preparation.

TEM is a powerful method for analysing nanomaterials due to its high spatial resolution (due to the shorter wavelength of electrons) and ability to directly image samples.⁸¹ However, the technique suffers from poor statistics due to the limitation of analysis area, making it unsuitable for bulk measurements.⁸²

1.3.3.2 Dynamic light scattering

Dynamic light scattering (DLS) measures the light scattering of particles in solution which gives information of their hydrodynamic radii. Particles in solution move under Brownian motion and their diffusion coefficient, D , can be related to their hydrodynamic radius (R_h) by the Stokes–Einstein equation (Equation 1.5).⁸³

$$R_h = \frac{k_B T}{6\pi\eta D} \quad 1.5$$

where k_B is the Boltzmann constant, T indicates the temperature at which the analysis is carried out, and η is the kinematic viscosity of the solvent. R_h indicates the hydrodynamic radius of the particles, defined as the radius of a hypothetical nonporous sphere that diffuses at the same rate as the particles being characterised.⁸⁴

During the analysis, a laser is passed through the solution and the light is scattered by the particles, with the change in intensity of scattered light measured as a function of time. The fluctuations in scattering intensity measured is directly dependent on the diffusion time of the scattering objects, and so their diffusion coefficients can be estimated.

DLS requires a very low sample amount, has short acquisition and analysis times, covers a wide dynamic range (nm to μm), and is compatible with a variety of solvent compositions.⁸¹ As a relatively inexpensive, benchtop apparatus, DLS is highly applicable in routine nanoparticle characterisation.

However, there are a few limitations which must be considered. Firstly, standard algorithms are poor when interpreting data collected for polydisperse populations of particles, with size estimations typically biased toward the larger population components.⁷⁷ In addition, due to the assumption of spherically shaped particles, calculated R_h values for non-spherical particles are likely to be inaccurate. Finally, it is crucial during sample preparation to ensure the solution is sufficiently dilute to avoid multiple scattering effects.⁸⁵

1.3.3.3 Electrophoretic light scattering/zeta potential

Zeta potential, ζ , is a measure of the “effective” electric charge on the surface of a nanoparticle. Electrophoretic light scattering is used to estimate the zeta potential of nanoparticles in suspension from their electrophoretic mobility, μ_e (Equation 1.6).⁸³

$$\mu_e = \frac{v}{E} \quad 1.6$$

where v is particle velocity and E is the strength of the externally applied electric field. The zeta potential and the electrophoretic mobility are then related by the Henry equation (Equation 1.7).⁸¹

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0\zeta f(Ka)}{3\eta} \quad 1.7$$

where ε_r is the relative permittivity of the solution, ε_0 is the permittivity in vacuum, $f(Ka)$ the Henry function, and η the viscosity of the solution.

As with DLS, a laser is transmitted through the samples as an electric field is applied to the particle suspension. If the particles are charged, they experience a movement toward the electrode with the opposite charge to their surface charge. The movement of the particles induces a shift in frequency in the scattered light, which is proportional to the particle velocity.⁸⁶ The shift is then used to estimate the electrophoretic mobility of the particles in suspension and, in turn, their zeta potential.

Several parameters affect the zeta potential of particles in solution, namely the ionic strength of the solvent, the presence of charged or uncharged molecules that can adsorb on the particle surface, and the pH of the solution.⁸³

1.3.3.4 Small angle X-ray scattering

Small-angle X-ray scattering (SAXS) is a technique which measures the difference in X-ray scattering between regions of differing electron density to generate contrast.⁸⁷ Using a monochromatic beam of hard X-rays, the elastic scattering behaviour of the X-rays as it travels through the material is analysed at small angles (typically 0.1-10°). The resulting curves are

then fitted to appropriate models to determine particle properties such as size, size distribution, shape, aggregation number and core diameter.⁸⁴

When compared to TEM, SAXS is non-destructive, and provides structural data averaged over macroscopic sample volumes meaning results are more statistically average than TEM imaging.⁷⁷ With the highly tuneable setups at modern synchrotrons, SAXS is also capable of structural characterisation in realistic sample conditions.⁸⁷ This is of particular importance for nanoparticle research, where SAXS is routinely used to study particles in their native, solution environments, which can be challenging when using TEM. When combined with short (picoseconds to seconds) data collection times, SAXS becomes ideally suited for *in situ* experiments.⁸⁸

1.4 Polymersomes as biomimetic structures

Phospholipids are biological molecules that can encapsulate both hydrophobic and hydrophilic species within their self-assembled structures (liposomes). However, their poor colloidal and chemical stability limits their use in a laboratory setting. They also exhibit limited scope for modification and suffer from inherent high permeability.⁸⁸ As previously discussed, advancements in controlled polymer synthesis have enabled the development of self-assembled nanostructures from amphiphilic block copolymers with a range of morphologies. Polymer vesicles, known as polymersomes, are of particular interest due to their ability to mimic the compartmentalisation properties of liposomes.

These artificial structures have several advantages over traditional liposomes, such as size distribution control, enhanced chemical diversity and improved stability against dilution (Figure 1.17).⁹⁰ In addition, incorporation of certain functionalities can lead to polymersomes

that are responsive to a variety of stimuli (e.g. temperature, pH, light, redox, etc.).⁹¹ Combined with their capacity to confine molecules, these properties make polymersomes excellent candidates for use as catalytic nanoreactors, drug delivery systems, cellular models, and nanosensors.^{2, 28, 29, 92}

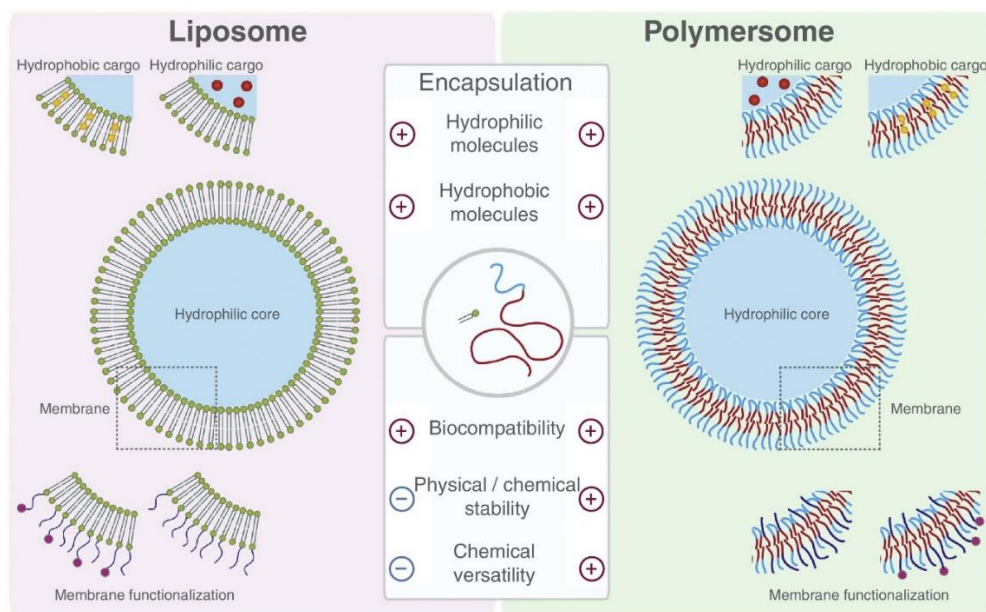


Figure 1.17. Schematic representation of liposomes (left) versus polymersomes (right). The key properties offered by each structure are shown in the centre. Adapted from Messenger *et al.*⁹³

The high molecular weight constituents of the polymersome bilayer membrane not only endow the structure with robustness and stability, but also with an increased barrier against diffusion, in comparison to their biological counterparts. If utilised effectively, this characteristic grants polymersomes a crucial advantage over liposomes in many applications.⁹⁴

1.4.1 Polymersome fusion

As a key mechanism utilised in material trafficking in biology, developing polymersomes with the ability to fuse and mix their interior compartments is of great interest in the development

of biomimetic systems. Although lipid bilayers in biology often require the action of SNARE proteins to overcome the high energy barrier for fusion, dissipative particle dynamics (DPD) simulations have shown that fusion can occur spontaneously when two conditions are met: (1) the particles maintain close contact and (2) there is sufficient membrane tension to overcome the energetic barrier of fusion.⁹⁵

Using a biomimetic approach, scientists have employed biological machinery (e.g. SNARE proteins) to promote fusion in artificial vesicles comprised of phospholipids, known as liposomes.⁹⁶⁻⁹⁸ In addition, protein-free liposome fusion has also been realised through a variety of techniques, from simple physiochemical triggers, e.g. application of an electric field,⁹⁹ to more complex biomimetic strategies, such as metal-ligand complexation,¹⁰⁰ attractive interactions,¹⁰¹ and small molecule recognition.¹⁰²⁻¹⁰⁴

While liposome fusion mechanisms and their applications have been extensively investigated, only a limited number of studies investigating polymersome fusion have been reported.^{105, 106} These can be categorised as: (1) spontaneous fusion, where polymersomes fuse without external intervention; (2) induced fusion, where particles do not have significant membrane tension to fuse when first synthesised, but, upon an input of energy (e.g. mechanical, electrical), the activation energy to fusion is overcome, resulting in polymersome fusion; and (3) accelerated fusion, for which the inclusion of additional machinery (e.g. fusogenic proteins) facilitates the fusion of polymersomes, that would not spontaneously fuse in isolation, by reducing the activation energy to fusion (Figure 1.18).

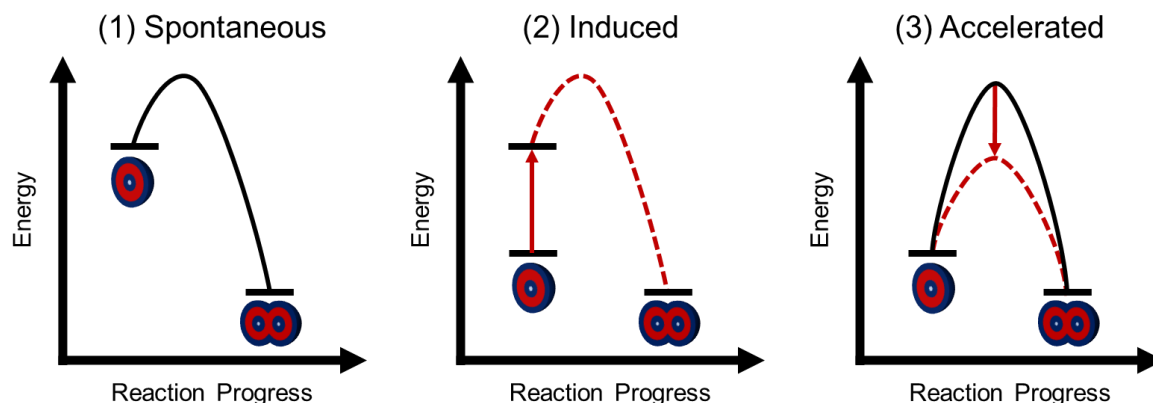


Figure 1.18. Hypothesised energy diagram for the three types of polymersome fusion: (1) spontaneous, (2) induced, and (3) accelerated.

Many reports of spontaneous fusion employ specific interactions between polymer chains, such as aromatic π - π interactions or hydrogen bonding.¹⁰⁷⁻¹¹⁰ These interactions induce specific packing arrangements of the polymers within the membrane resulting in an increased level of tension that can then overcome the energy barrier to fusion. For example, Zhu *et al.* generated nanotubes from the spontaneous fusion of spherical vesicles prepared by PISA.¹¹¹ They highlighted the importance of the aromatic interactions, originating from the π -stacking between anthracene side chains within the solvophobic block, in the formation of tubular structures. As such, inclusion of a weakly aromatic monomer, 2-(methacryloyloxy)ethyl benzoate (MAEB), only resulted in the formation of spherical vesicles.

Although it has been demonstrated with a range of polymersome systems and self-assembly methods, a critical feature of all spontaneously fusing polymersomes is the requirement for low chain mobility, especially with respect to the core-forming block.¹¹¹⁻¹¹³ For fluid membranes, such as those found in liposomes and naturally occurring biological vesicles, any increase in membrane tension can be released by chain rearrangement or unimer exchange, allowing the formation of structures lowest in free energy. In contrast, low chain mobility

allows the polymersomes to be formed in a kinetically trapped state, so that fusion becomes the preferred pathway over particle rearrangement.

As a representative example of this, Tan and co-workers explored how different pathways of morphological transformation operated at different temperatures in a photo-PISA system.¹¹⁴

At 60 °C, which was above the T_g of the core-forming block, continued polymerisation to higher core DPs led to inward and outward growth of the polymersome membrane. However, when the polymerisation was conducted at 25 °C, chain rearrangement was severely retarded. This led to a build-up of membrane tension as the polymer chain length increased and resulted in the fusion of particles, producing lower energy tubular structures. It is the difference in relative rates of polymerisation and self-assembly at the two temperatures that causes the variance in final nano-object morphology.

Moreover, O'Reilly and co-workers demonstrated the fusion of ROMPISA polymersomes above a critical core DP, forming elongated tubular structures (Figure 1.19).¹¹⁵ Initially, they showed that fusion was prohibited in acidic solutions for polymersomes containing an amine group in the corona, due to the electrostatic repulsion between particles that results in an increased activation energy for fusion. However, uncharged polymersomes, with a polyethylene glycol (PEG) based corona, were shown to fuse above a critical core DP. In addition, it was shown that tube length increased with respect to core block length, highlighting the link between membrane tension and particle fusion, as seen in other reports.^{110, 116} One-dimensional (1D) fusion was a predominant feature across several polymersome fusion studies, with vesicle fusion deviating from this considered as

unfavourable due to the high membrane curvature still present in the resulting fused structures.^{111, 113}

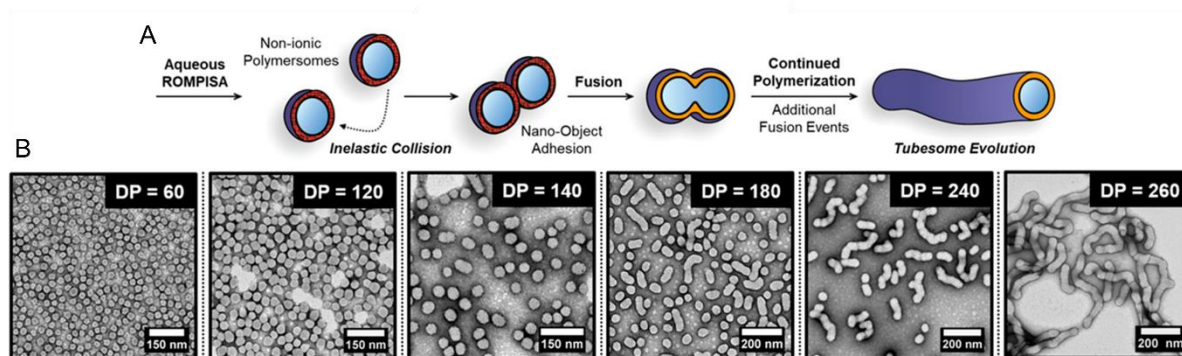


Figure 1.19. (A) Schematic representation of tubesome evolution *via* polymerisation-induced polymersome fusion. (B) Dry-state TEM images of the various morphologies formed at different core DPs. Adapted from Varlas *et al.*¹¹⁵

TEM analysis of the nanostructures reported by O'Reilly and co-workers possess a greater amount of uncertainty regarding their morphology than polymersomes synthesised using different methods (e.g. thin-film rehydration, solvent switch, or RAFT PISA) due to the lack of a distinguishable membrane and lumen.¹¹⁵ In addition, the small size of ROMPISA nanoparticles (typically < 50 nm), and their small section within the phase diagram, increases the difficulty in probing lower curvature morphologies (i.e. spheres and worms).

However, additional static light scattering (SLS) analysis by O'Reilly and co-workers provides further evidence that the initial structures formed are polymersomes which subsequently fuse to form tubesomes. SLS analysis allows for the quantification of two parameters: radius of gyration (R_g) and hydrodynamic radius (R_h). The ratio of these parameters can then be used to determine morphology, with a solid sphere of uniform density having an R_g/R_h value of 0.78 whilst a value of 1 indicates a vesicle with an infinitesimally thin membrane.

As such, when DP of the core block was increased from 40 to 60, a decrease in the measured R_g/R_h value was observed (0.87 to 0.82), suggesting the structure is becoming more sphere-like. This result suggests the ROMPISA nanoparticles are a hybrid structure between a solid sphere and a hollow vesicle (i.e. a vesicle with a small lumen) and the increase in DP leads to an inward growth of the membrane. This inward membrane growth has also been observed in polymersomes synthesised by RAFT PISA.¹¹⁷

Whilst the aforementioned examples successfully demonstrate fusion, it would also be desirable to achieve temporal control, comparable to that achieved in biology. As such, there are several examples of modified polymers that have been designed to fuse upon application of an external stimulus. For example, Zhang and co-workers reported the fusion of poly(*N*-isopropylacrylamide)-*b*-poly(6-[4-(4-methylphenyl-azo) phenoxy] hexylacrylate) (PNIPAM-*b*-PAzoM) polymersomes upon exposure to UV light.¹¹⁸ The photo-responsive azobenzene units contained within the polymer chain undergo a *trans*-to-*cis* isomerisation when exposed to light at 365 nm. The authors suggest that, following isomerisation to the *cis* isomer, the azobenzene units became better solubilised in the exterior solution, causing a shift in the hydrophilic/hydrophobic balance that destabilises the vesicle membrane and promotes fusion. In addition, they also highlight the possibility that disruption in the polymer packing, as a result of the photoisomerisation process, may cause deformations within the membrane and drive particle fusion.

Several methods have been employed to induce fusion in polymersomes, including agitation,¹¹⁹ osmotic pressure,¹²⁰ and ultrasound.¹²¹ In induced fusion, polymersomes can be combined in solution but do not fuse until an energy input is applied. This leads to a

destabilisation of the polymersome membrane, forming a metastable state, and means fusion is now a favourable process. However, because energy is added to the system, the membranes do not need to be rigid, as with spontaneous fusion. As such, the higher chain mobility results in greater chain rearrangement during fusion and leads to the formation of spherical polymersomes as a final product. Due to the fluidity in the membrane, leakage of the internal cavity is often reported for induced fusion studies.¹²¹

The final strategy to control polymersome fusion is to employ additional machinery, for example fusogenic proteins. This direct, biomimetic strategy has been achieved by Otrin and co-workers using a grafted copolymer containing PDMS and PEG, functionalised with fusogenic SNARE proteins.¹²² As well as examining the mechanism of fusion through extensive cryo-EM imaging, the researchers also examined the efficacy of full fusion between polymersomes. This was achieved by preparing two SNARE-functionalised vesicle populations: one containing ATP synthase (AS) and the other the enzyme *bo*₃ oxidase. AS couples adenosine diphosphate (ADP) and inorganic phosphate, in the presence of protons, to produce ATP. When *bo*₃ oxidase is reduced by ubiquinol, protons are translocated across the membrane. When both enzymes are integrated into a single compartment, ATP is synthesised (Figure 1.20). As such, when the two vesicle populations were mixed, a steady state of ATP production was observed, indicating full SNARE-mediated membrane fusion had taken place.

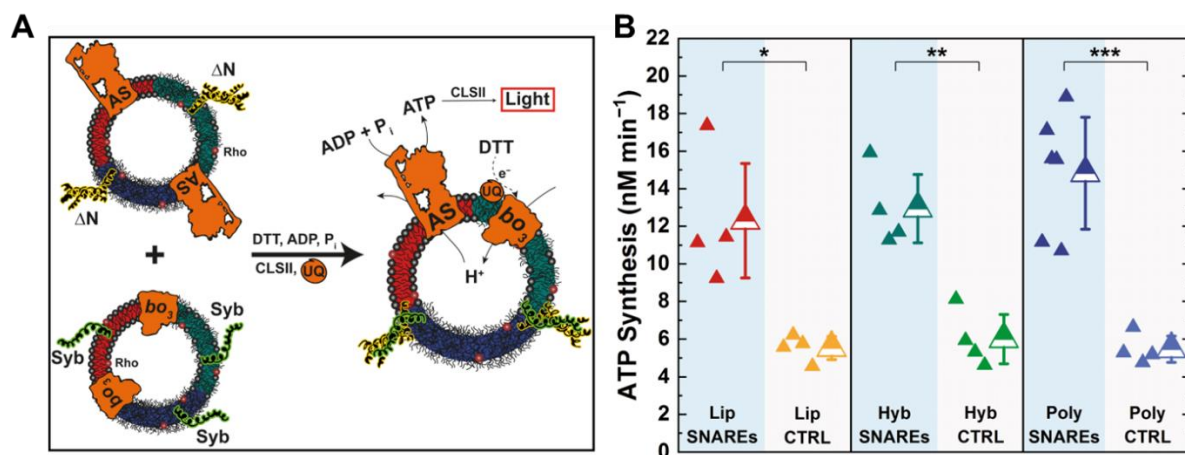


Figure 1.20. (A) Schematic representation of content mixing assay utilising the proton pump *bo3* oxidase (*bo3*) and the proton gradient consumer ATP synthase (AS). (B) Comparison of ATP synthesis rates after SNARE-free or SNARE-mediated fusion of different vesicles. Adapted from Otrin *et al.*¹²²

With the aim of replicating the temporal control of biological fusion, Fielden *et al.* demonstrated the two-step triggered polymersome fusion of triblock polymersomes synthesised *via* aqueous ROMPISA.¹²³ The fast polymerisation rate of ROMPISA and the rigidity of the norbornene backbone leads to the formation of metastable structures with high membrane tension (like those formed in spontaneous fusion). However, the incorporation of a short, amine-functionalised block into the polymer chain inhibited particle fusion at pH 2 due to the electrostatic repulsion of the protonated amine groups. Upon addition of base to switch the solution to pH 12, particle fusion was triggered to form elongated tubular structures, demonstrating the temporal control of the fusion process.

1.4.2 Encapsulation of cargo

Encapsulation is perhaps the most important property that dictates the potential applications of a polymersome system.⁹⁴ In drug delivery systems, the polymersome must transport a therapeutic agent to its target location whilst simultaneously protecting it from

decomposition.¹²⁴ Additionally, nanoreactors can be synthesised by encapsulating a catalyst with a polymersome, increasing its efficiency through enhanced local concentration of reagents.⁹⁰

The encapsulation of cargo within polymersome structures, either during or after polymer self-assembly, has been widely reported.¹²⁵ Due to the structure of polymersomes, there are a number of possible locations for cargo to be encapsulated (Figure 1.21).¹²⁶

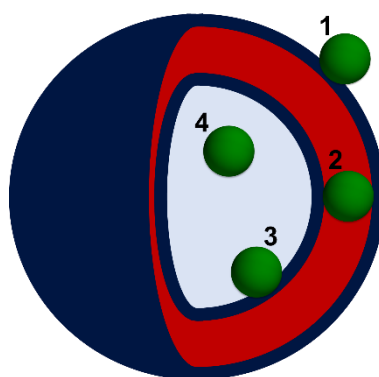


Figure 1.21. Schematic visualisation of possible locations of encapsulated cargo within the polymersome: inner (3) and outer (1) hydrophilic surfaces, the hydrophobic membrane (2) or the lumen (4). Adapted from Gumz *et al.*¹²⁶

The ability for polymersomes to encapsulate cargo is typically described as the encapsulation efficiency (%EE) and is defined as:

$$\%EE = \frac{m_{encapsulated}}{m_{total}} \times 100 \quad 1.5$$

where $m_{encapsulated}$ is the mass of encapsulated material and m_{total} is the total mass of material added to the system.

Due to the reduced permeability compared to liposomes, polymersomes typically display higher encapsulation efficiencies for water-soluble cargos.⁸⁹ Multiple studies focussed on

quantifying and/or improving the encapsulation efficiency of polymersome systems have been reported, employing a range of analytical techniques, such as electron microscopy, confocal microscopy and fluorescence spectroscopy.¹²⁷⁻¹³⁵ Different cargos have been encapsulated within synthetic vesicles, from simple small molecules through to complex biomolecules, such as enzymes and proteins.^{90, 93, 136, 137}

1.5 Summary

In this chapter, compartmentalisation in nature and its key benefits have been outlined. The different mechanisms by which material is transported throughout biological systems were described, including passive, active, and bulk transport. Some examples of biomimetic strategies to replicate these mechanisms have been highlighted.

Moreover, the key concepts of polymer synthesis were introduced, with particular focus on techniques used in the preparation of block copolymers. This included an introduction to ROMP, emphasising fundamental features in its mechanism, such as the release of ring-strain as a thermodynamic driving force and the formation of a metallacyclobutane intermediate.

Thereafter, a detailed description of the self-assembly of amphiphilic block copolymers was provided, including empirical rules relating the packing parameter nanoparticle morphology. The advantages of ROMPISA compared to other self-assembly methods were discussed, including rapid polymerisation kinetics and polymer backbone rigidity resulting in the formation of kinetically trapped morphologies. Aqueous ROMPISA was used for the preparation of block copolymer nano-objects throughout this thesis. A short introduction to the techniques used for the analysis of polymer self-assemblies was outlined.

Finally, the applicability of polymersomes as biomimetic structures was discussed, focussing on polymersome fusion and encapsulation of cargo. Fusion mechanisms of ROMPISA nano-objects were investigated in Chapter 2 and 3, whilst Chapter 4 explored the encapsulation of water-soluble cargo.

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Chapter 1

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Chapter 2. Expanding the Scope of pH- Triggered Fusion of Polymersomes Synthesised *via* Aqueous Ring-Opening Metathesis Polymerisation Induced Self-Assembly

2.1 Abstract

Biological fusion of phospholipid membranes is a highly regulated process that controls the movement of material throughout a variety of structures. Often triggered by an external stimulus, the fusion of phospholipids can be coordinated with a multitude of other processes (e.g. nerve propagation). In contrast, the fusion of polymer-based membrane bound structures (polymersomes) is typically spontaneous, caused by an increase in membrane tension during formation that cannot be released by simple chain-rearrangement. In contrast, out-of-equilibrium polymersomes prepared by ring-opening metathesis polymerisation induced self-assembly (ROMPISA) have been shown to fuse upon application of a trigger (pH change).

Herein, we demonstrate the scope of this triggered fusion process through the incorporation of a carboxylic acid-functionalised monomer. A variety of conditions for polymersome synthesis are explored, as well as the effect of trigger concentration on the resulting nano-object morphology. Kinetic analysis of polymersome fusion following a pH switch allows for the elucidation of mechanistic insights and directional selectivity. Dual, pH-responsive quadrablock polymersomes containing both carboxylic acid- and amine-functionalised monomers are synthesised. The impact of block order and polymer microstructure on the triggered fusion of these polymersomes is also explored. These results provide an enhanced understanding of ROMPISA triggered fusion and promotes further development of synthetic systems with biomimetic fusion properties.

2.2 Introduction

Compartmentalisation of molecules into distinct environments is the key to nature's structural organisation.¹ There are many benefits to compartmentalisation within living cells, including protecting sensitive entities (e.g. genetic material) from degradative processes and increased reaction rates from high local concentrations of reagents.² However, these benefits are conditional on the ability to regulate the movement of chemicals into and out of these compartments.

Critical to a multitude of biological processes, the transportation of material in and around cellular compartments is governed by the fusion of phospholipid bilayer membranes.³ This process is strictly regulated in biology because phospholipid fusion is not typically a spontaneous process.⁴ There is a large energetic barrier to fusion, owing to the electrostatic repulsion of charged head groups, as well as the resulting increase in tension as the membrane deforms.

As such, phospholipid membrane fusion is mediated by specific interactions between fusion proteins, termed SNAREs (Figure 2.1).⁵ These membrane-bound proteins are situated on the two membranes undergoing fusion. Initially they tether the membranes together by forming complementary interactions with the proteins on the opposing membrane. This tethering induces deformations within the phospholipid membrane, thus increasing tension and producing a metastable structure. The application of an additional stimulus (e.g. Ca^{2+} ions) leads to the collapse of this metastable structure and promotes the fusion of the membranes. This two-step process allows for specificity in membrane fusion, that can also be coupled with other processes (e.g. electrical nerve signals).⁶

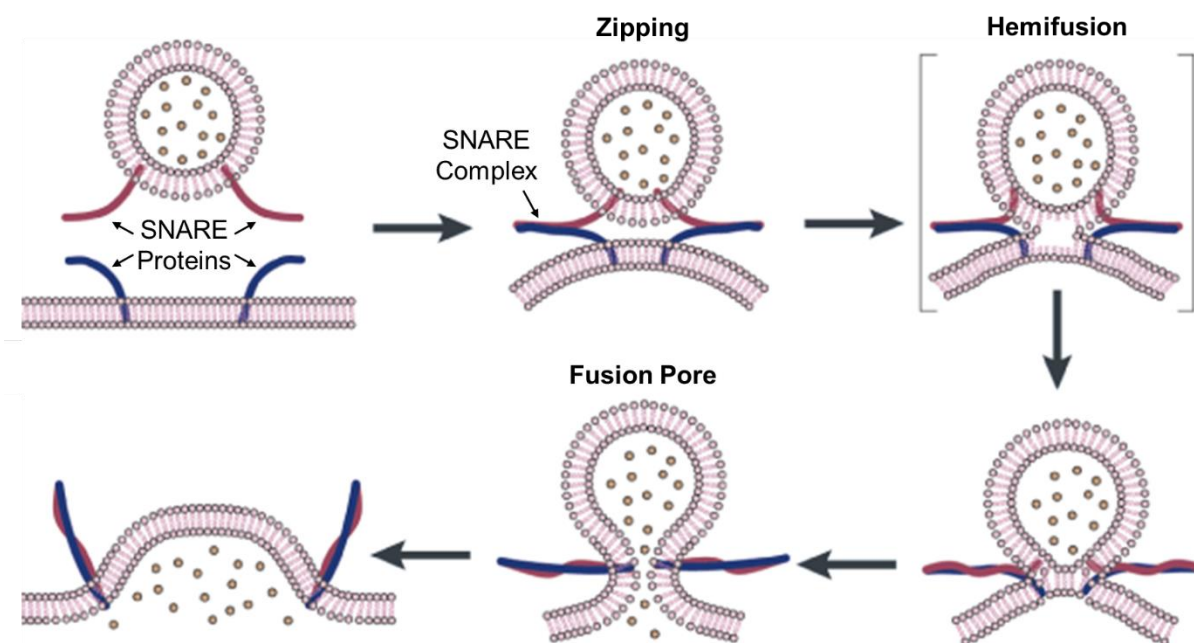


Figure 2.1. Schematic representation of a SNARE-mediated membrane fusion process. Adapted from Chen *et al.*⁷

In biology, machinery can be used to achieve vesicle fusion with exquisite selectivity with no external input of energy. If we can design a wholly synthetic system that takes inspiration from this, then we can develop nanotechnology with much greater sophistication than the current state of the art. For example, we could create vesicles that fuse in response to chemical reactions (i.e. a product of a reaction causes fusion), vesicles that operate in organic solvents, and cascades of fusion that allow elaborate reaction sequences to occur in an autonomous system.

To this aim, researchers have used a variety of methods to create systems of artificial phospholipid vesicles, known as liposomes, with the ability to fuse. For example, directly

utilising biological machinery through incorporation of SNARE proteins into liposomes has been reported as an efficient methodology to obtain temporal control of fusion.⁸

Moreover, biomimetic strategies such as hydrogen bonding and small molecule recognition have also been employed to control the fusion of liposomes.⁹⁻¹¹ Lehn and co-workers synthesised lipids bearing either barbituric acid (BAR) or 2,4,6-triaminopyrimidine (TAP) groups, which form a complementary pair involving both hydrogen bonding and electrostatic forces (Figure 2.2).¹² Each complementary lipid was combined with egg lecithin (EPC) to form two populations of large, unilamellar liposomes. When mixed in a 1:1 ratio, the researchers observed an increase in turbidity within minutes, which was further supported by light scattering analysis indicating an increase in hydrodynamic diameter from 150 to 2000 nm. However, fusion occurred immediately once the liposomes were mixed, preventing the system from being triggered to an external stimulus.

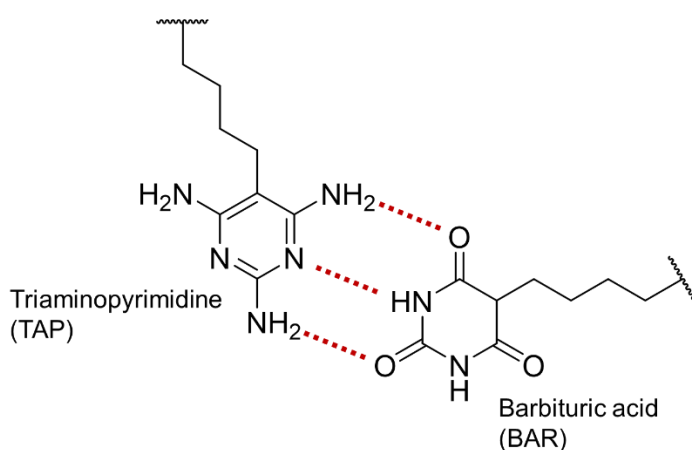


Figure 2.2. Chemical structures of barbituric acid (BAR) and 2,4,6-triaminopyrimidine (TAP) and their associated hydrogen bonding interactions.

As a step towards triggered fusion, synthetic chemistry has also been incorporated to mediate fusion in liposomes. Dimova and co-workers produced lipid vesicles functionalised with

amphiphilic β -diketone ligands, molecules that are known to be cross-linked by Eu^{3+} ions (Figure 2.3).¹³ When two liposomes were brought into close proximity to one another and exposed to a small volume of EuCl_3 at the area of contact, fusion of the liposomes was observed by optical microscopy over a timescale of <100 ms (Figure 2.3). In contrast, in the absence of the β -diketone groups, non-specific interactions between the lipid molecules in the membrane and Eu^{3+} induced adhesion but no fusion.

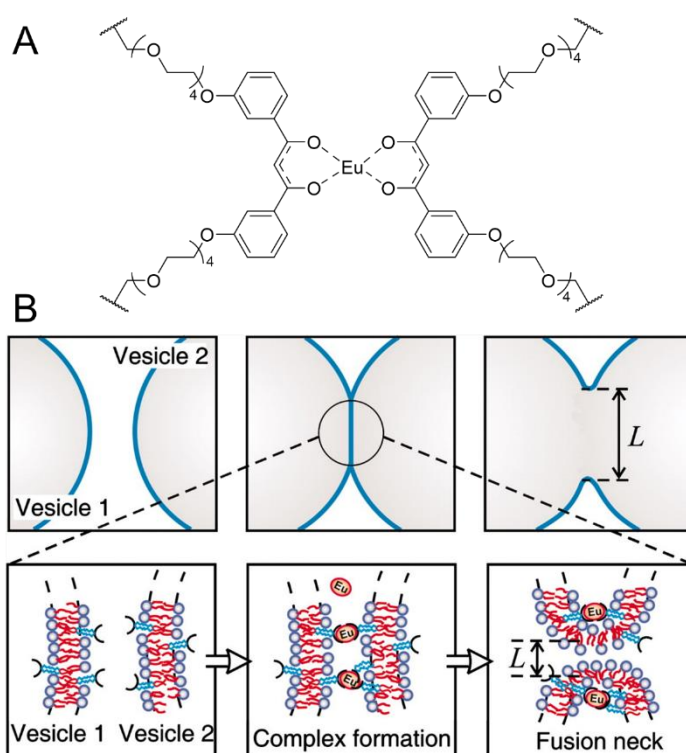


Figure 2.3. (A) Chemical structure of two β -diketone ligands forming a complex with an Eu^{3+} ion. (B) Schematic diagram of the fusion process between two β -diketone-functionalised liposomes when triggered with EuCl_3 . Adapted from Haluska *et al.*¹³

Another example of triggered liposome fusion utilises click chemistry to fuse vesicles, termed clickosomes.¹⁴ Zumbuehl and co-workers synthesised two complementary diether phospholipids bearing either azide or alkyne pendant groups which, in Cu(I) -catalysed 1,3-dipolar cycloaddition conditions, react to form a triazole bridge. (Figure 2.4).

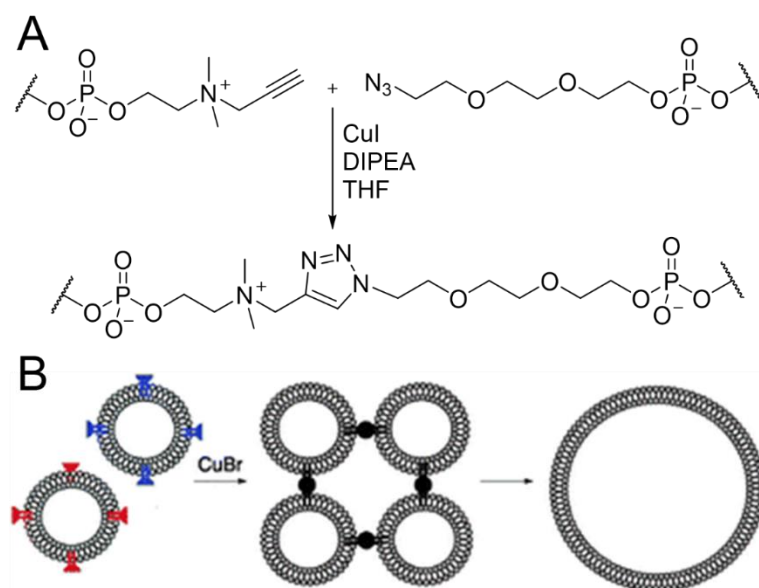


Figure 2.4. (A) Reaction scheme for the formation of a triazole-bridged phospholipid by a Cu(I)-catalysed Huisgen–Sharpless click reaction between alkyne- and azide-functionalised phospholipids. (B) Proposed two-step mechanism for the Cu click-mediated fusion of liposomes. Adapted from Loosli *et al.*¹⁴

The functionalised phospholipids were successfully incorporated into separate liposome populations and mixed in equimolar mounts. Upon addition of CuBr, the mixture showed vesicle aggregates after one day, as measured by DLS, and, after four days, vesicle fusion was observed by TEM. In addition, no fusion was observed when CuBr was not added or one of the complementary phospholipids was omitted. The authors proposed a two-step mechanism whereby the oppositely charged liposomes first aggregate and are tethered together by the 1,3-dipolar cycloaddition. Following this, the close proximity of the membranes promotes vesicle fusion into larger vesicles (Figure 2.4).

While liposome fusion with selectivity and temporal control has been extensively studied, designing the analogous ability in polymersomes is an underexplored area. In addition, the majority of polymersome fusion studies employ the mechanism of induced fusion in which an

energy source (e.g. sonication, UV light) is employed to lower the activation energy and promote fusion. Although successful, the requirement for an external energy source prevents the synthesis of a highly specific and autonomous system.

As an alternative to induced fusion, several polymersome systems that undergo fusion spontaneously have been developed. This is where polymersomes are synthesised with significant membrane tension which can overcome the thermodynamic barrier to fusion. Membrane tension is built up when individual polymer chains are unable to rearrange themselves into a lower energy arrangement during self-assembly. This may be due to a restriction in chain mobility or interactions between polymer chains (e.g. hydrogen bonding or π - π interactions) resulting in specific packing arrangements. Unable to lower their energy levels by chain rearrangement, the polymersomes release the energy stored as membrane tension through fusion.

An alternative method to produce nano-objects with high membrane tension, taking advantage of the rapid nature of ROMP and rigidity of polynorbornene, has been developed by O'Reilly and coworkers.¹⁵ Aqueous ROMPISA utilises the simultaneous polymerisation and self-assembly of substituted norbornene monomers using a ruthenium-based catalyst to form nano-sized structures. A study employing several core-forming monomers demonstrated that polymersomes of similar size are formed across a wide range of target core DPs.^{16, 17} This is in contrast to polymersomes obtained by alternative PISA methodologies (e.g. RAFT) which generally increase in size as the core DP is increased.¹⁸ The rod-like nature of polynorbornene results in a high T_g and consequent low chain mobility, especially in aqueous solution, resulting in the formation of high membrane tension polymersomes.

As such, in a follow up study, O'Reilly and coworkers demonstrated the fusion of ROMPISA polymersomes above a critical core DP, forming elongated tubular structures.¹⁹ The researchers synthesised an amphiphilic diblock copolymer comprised of a hydrophilic PEG-functionalised polynorbornene (P(**NB-PEG**)) and a hydrophobic ether-functionalised polynorbornene (P(**NB-MEG**)) which self-assembles in aqueous solution. At low DP, self-assembly results in the formation of small spherical polymersomes. However, as DP of the hydrophobic block increases, the polymer chains are unable to undergo chain rearrangement due to the rigidity of the unsaturated, ring-bearing polymer backbone. This results in a build-up of membrane tension which, above a critical DP, is then released during fusion.

In addition, the researchers also demonstrated that polymersome fusion could be perturbed by the incorporation of a charged functionality within the hydrophilic block. An amine-functionalised polynorbornene (P(**NB-Amine**)) is protonated in the acidic conditions used for ROMPISA, and thus, when used as the hydrophilic block, resulted in positively charged polymersomes. The addition of an electrostatic repulsion between polymersomes resulted in no fusion until the core DP was increased to 420. At this point, the membrane tension was high enough to overcome both the electrostatic repulsion between polymersomes, as well as the energetic barrier to fusion.

Due to the spontaneous nature of ROMPISA polymersome fusion, this is unable to be coordinated to other processes, as is often observed in nature. Instead, structures that inherently have the energy required to fuse (and thus do not require an external energy source) but are prohibited to do so prior to a trigger being applied (endowing the system with temporal control) are of great interest.

As a seminal example (which I build upon in this chapter), recent work by Fielden *et al* demonstrated the two-step triggered polymersome fusion of triblock polymersomes synthesised *via* aqueous ROMPISA (Figure 2.5).²⁰ Knowing that electrostatic repulsion can restrict fusion in polymersomes, the researchers incorporated a short P(**NB-Amine**) block between the hydrophilic and hydrophobic blocks, resulting in the formation of positively charged spherical polymersomes with high membrane tension. The polymersomes were unable to release their energy by fusion or chain rearrangement, and thus remained in a metastable state.

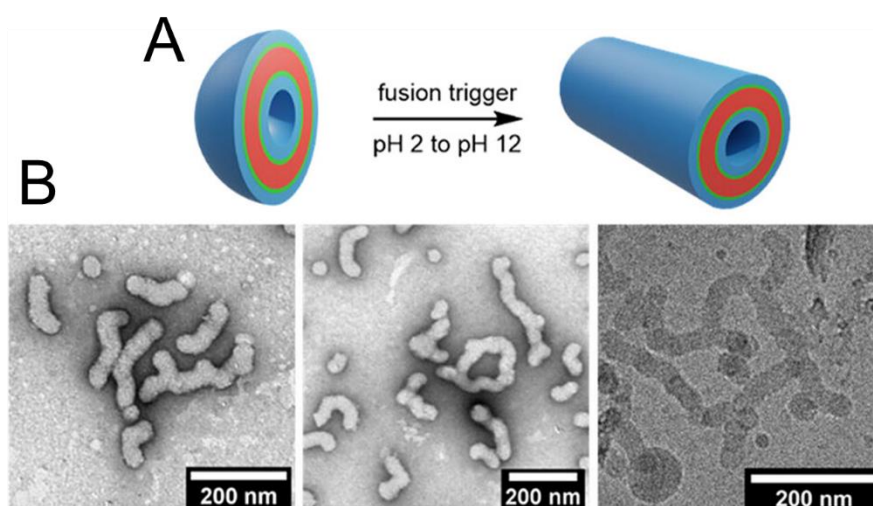


Figure 2.5. Schematic diagram of triggered polymersome fusion of triblock polymersomes by a pH switch and TEM images of the resulting nano-objects. Adapted from Fielden *et al.*²⁰

Upon addition of base to switch the solution from pH 2 to 12, an immediate increase in solution turbidity was observed. In addition, an increase in hydrodynamic diameter and the observation of fused structures by TEM analysis supports the successful fusion of the polymersomes upon application of a pH trigger. The authors suggest that the P(**NB-Amine**) block was deprotonated upon pH switch, thus becoming neutral and hydrophobic. The

removal of electrostatic repulsion between polymersomes, as well as the change in hydrophilic/hydrophobic ratio, lowers the activation energy of fusion, making it a thermodynamically favourable process driven by membrane tension.

Control experiments evidenced that fusion was specifically triggered by a change in the protonation state of the P(**NB-Amine**) block, as opposed to a change in reaction conditions, such as dilution or agitation. Further complementary analysis by *in situ* SAXS was used to elucidate that fusion proceeded by a two-step mechanism: (1) rapid aggregation to give elongated particles; (2) structural rearrangement to give the final fused product.

This initial work on triggered polymersome fusion had limited scope, requiring specific conditions and a large pH change to induce fusion. Herein, we demonstrate the generality of the triggered fusion process using polymersomes synthesised by ROMPISA. The polymerisation of carboxylic acid-functionalised triblock copolymers and the formation of anionic, self-assembled nanostructures was achieved. In addition, the influence of buffer pH on the resulting nano-object morphology was investigated, whilst the capacity to form colloidally stable, anionic nanostructures in a neutral, saline solution was also established.

Following this, the triggered fusion of carboxylic acid-functionalised triblock polymersomes by a switch to acidic pH was demonstrated. Kinetic analysis of the triggered fusion process provided useful mechanistic insights and allowed for the extrapolation of directional selectivity afforded by carboxylic acid containing polymersomes compared to their amine-functionalised analogues.

Finally, the inclusion of both amine- and carboxylic acid-functionalised norbornene monomers within quadrablock copolymers, and their self-assembly into zwitterionic ROMPISA

polymersomes was also achieved. Due to the presence of two pH-responsive functional groups within the nano-objects, the triggered fusion of particles by either an increase or decrease in solution pH was exhibited, with the impact of block order and polymer microstructure on this process also explored. Lastly, the ability of these polymersomes to undergo stepped, dual pH-responsive fusion was investigated.

2.3 Results and Discussion

2.3.1 Triggered polymersome fusion of carboxylic acid-containing triblock nano-objects synthesised *via* ROMPISA

Analogous with previous reports, a polyethylene glycol substituted norbornene monomer (**NB-PEG**) was chosen as the corona monomer because it is uncharged and remains hydrophilic across different solution pH. In addition, norbornene imide ethylene glycol monomethyl ether (**NB-MEG**) was also used as the hydrophobic core forming monomer as it is pH unresponsive and simple to synthesise. Finally, a modified Grubbs' 3rd generation catalyst (**G3**) was used as it provides fast initiation and polymerisation rates as well as being easily accessible from a commercially available starting material (Figure 2.6).

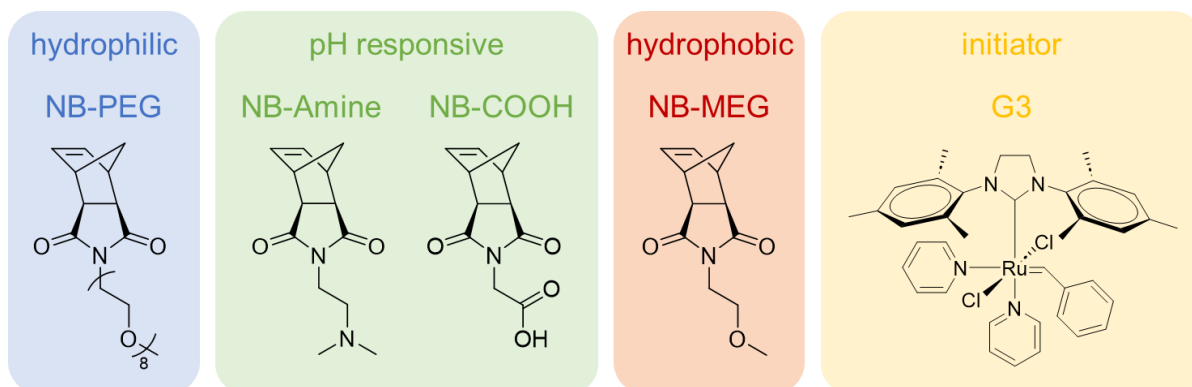


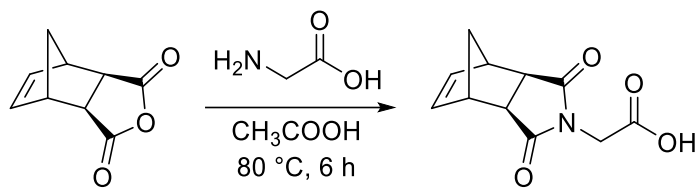
Figure 2.6. Chemical structures of monomers and initiator used in this chapter.

An amine-functionalised monomer (**NB-Amine**) has previously been demonstrated to be pH-responsive and was utilised in pH triggered polymersome fusion.²⁰ For this work, a carboxylic acid-functionalised monomer was chosen to be utilised within polymersomes that would respond to a decrease in solution pH, inverse to the amine-containing polymersomes. Previously, O'Reilly and coworkers reported the use of *exo*-5-norbornene carboxylic acid as a core monomer in the formation of nano-objects with different morphologies *via* ROMPISA.¹⁵

Norbornenes are typically hydrophobic in nature because of their chunky hydrocarbon backbone. However, ROMP monomers containing an imide anchor group are more hydrophilic than those with either an ester or amide linkage, when the same terminal group was present.¹⁷ As such, the addition of an imide anchor group improves the solubility of the norbornene in aqueous solution and allows access to corona-forming monomers.

Research has also shown that the stereochemistry of the substituents on the norbornene ring affect the polymerisation rate when using a ruthenium-based catalyst. Rule and Moore compared the reaction rates of *endo*- and *exo*-dicyclopentadiene (DCP) in ROMP when using a Ru-based catalyst.²¹ They observed that *exo*-DCP was nearly 20 times more reactive than *endo*-DCP at room temperature. This was primarily attributed to steric interactions between the approaching monomer and the substituents of the cyclopentane ring to which the propagating centre is bound. This steric interaction is far greater in *endo*-DCP, reducing the rate of polymerisation compared to the *exo* form.

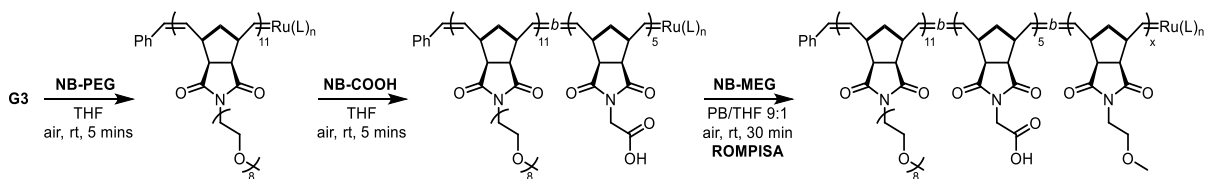
The rapid polymerisation kinetics during ROMPISA prevent chain rearrangement from occurring during self-assembly, forming kinetically trapped polymersome structures with high membrane tension.¹⁹ In order to maintain this fast polymerisation rate, an *exo*-norbornene imide structure was chosen as the basis for the carboxylic acid containing monomer (**NB-COOH**). The synthesis of *exo*-Norbornene imide carboxylic acid (**NB-COOH**) was performed according to a previously reported protocol (Scheme 2.1).²² The synthesis was achieved *via* an intramolecular condensation reaction between *exo*-norbornene anhydride and glycine, followed by an acidic work up to ensure the product was uncharged, with a yield of 81% (Figure S2.1 and S2.2).



Scheme 2.1. Synthesis of *exo*-Norbornene imide carboxylic acid (**NB-COOH**).

2.3.1.1 Synthesis and characterisation of carboxylic acid-containing triblock nano-objects *via* ROMPISA at pH 2

To explore the effect of incorporating **NB-COOH** into polymersomes, the amphiphilic block copolymers, **P_{pH2}1_x**, **P(NB-PEG)₁₁-*b*-P(NB-COOH)₅-*b*-P(NB-MEG)_x**, containing a corona formed from discrete blocks of **NB-PEG** with **NB-COOH** and a core of **NB-MEG** (DP = *x*) were accessed using ROMPISA (Scheme 2.2). This was performed analogously to a previously reported method of polymersome synthesis.²⁰ To produce nano-objects, the corona was first synthesised by reacting the **G3** initiator with the hydrophilic monomers, first **NB-PEG** and then **NB-COOH** in tetrahydrofuran (THF). The resulting water-soluble macroinitiator was then chain-extended upon addition of **NB-MEG** dissolved in 100 mM phosphate buffer (PB) adjusted to pH 2 to give a final solvent composition of THF/PB2 of 1:9 v/v.



Scheme 2.2. Synthetic route for the preparation of **PpH21_x** block copolymers by ROMPISA at pH 2.

An increase in turbidity was observed for all solutions, with **P_{pH2}1₃₀₀** forming a free standing hydrogel as determined by the vial inversion test (Figure S2.3).²³ Proton nuclear magnetic resonance (¹H NMR) spectroscopy was used to determine complete consumption of **NB-MEG** within 30 minutes, as shown by the loss of peaks at 6.3 ppm (Figure S2.6, S2.7 and S2.8). SEC

analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.2$) for all core DPs (Table 2.1 and S2.1 and Figure 2.7).

Table 2.1. Characterisation summary of $\mathbf{P_{pH2}1_x}$.

Polymer	$M_{n,theo}$ / kDa	$M_{n,SEC}$ / kDa	$\mathcal{D}_{SEC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
$\mathbf{P_{pH2}1_{100}}$	29.7	33.8	1.18	42	0.20	29 ± 1
$\mathbf{P_{pH2}1_{200}}$	51.8	58.2	1.19	76	0.12	N/A ^a
$\mathbf{P_{pH2}1_{300}}$	73.9	86.9	1.22	358	0.39	N/A ^a

^adry-state TEM image analysis was not possible due to the ambiguous start and end points of the fused particles.

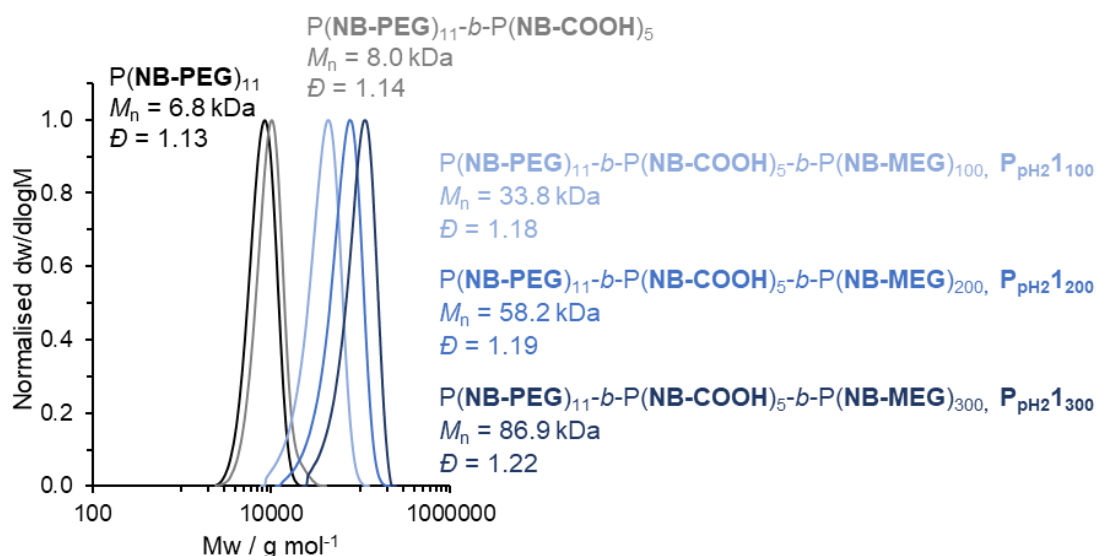


Figure 2.7. Normalised SEC traces (THF eluent, PMMA standards) of $\mathbf{P_{pH2}1_x}$ and intermediate polymers.

The resultant nano-objects were analysed by DLS which showed increasing average hydrodynamic diameters (42, 76 and 358 nm) as the DP of the core block was increased from 100 to 200 and then 300, respectively (Table 2.1 and Figure 2.8). TEM analysis showed spherical polymersomes for $\mathbf{P_{pH2}1_{100}}$ and fused tubes for $\mathbf{P_{pH2}1_{200}}$ and $\mathbf{P_{pH2}1_{300}}$, confirming that,

at low pH, the carboxylic acid-functionalised block is uncharged and therefore hydrophobic, resulting in the spontaneous fusion of polymersomes above a critical DP (Table 2.1 and Figure 2.9, S2.9 and S2.10). Due to the entangled nature of $P_{pH2}1_{200}$ and $P_{pH2}1_{300}$, image analysis was not possible to determine average tube length.

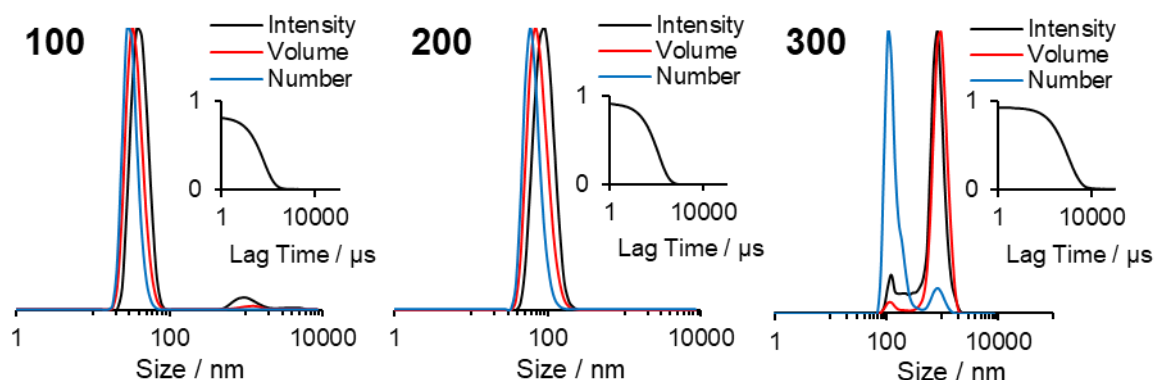


Figure 2.8. Normalised DLS analysis of $P_{pH2}1_x$ particles: $x = 100, 200$, and 300 .

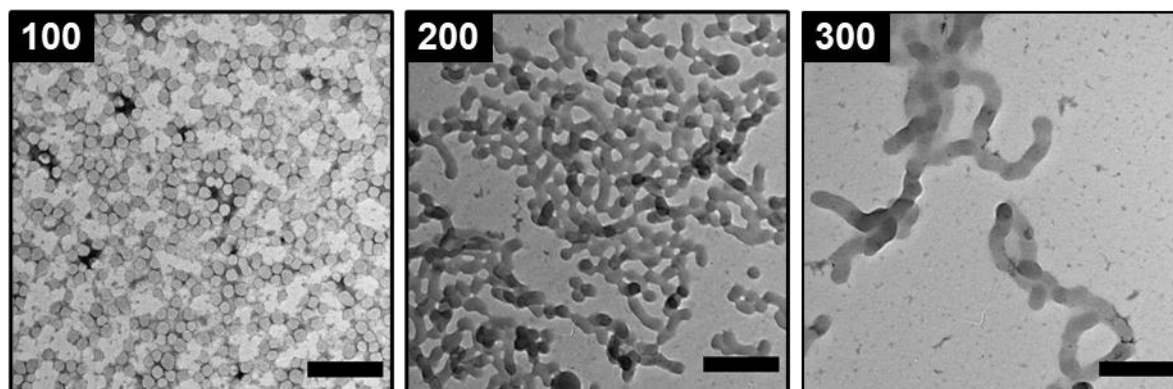


Figure 2.9. Dry-state TEM images of $P_{pH2}1_x$ particles: $x = 100, 200$, and 300 . Scale bar = 200 nm .

2.3.1.2 pH-dependent self-assembly of carboxylic acid-containing triblock nano-objects *via* ROMPISA

Above a critical DP, the carboxylic acid-containing polymersomes fuse to produce tubular structures at acidic pH. This is due to the protonation of the carboxylate group which eliminates the electrostatic repulsion between particles as well as switching the block from hydrophilic to hydrophobic. Carboxylic acids are weak acids ($pK_a \sim 4$) and so, when the pH is

adjusted, different self-assembly behaviour would be expected. Therefore, the switchable nature of the carboxylic acid moiety and its influence on the fusion process at different pH was explored.

The synthesis was performed using the same procedure as previously described but using 100 mM phosphate buffer solutions adjusted to pH 3, 4.5 and 7. This range of buffer pH will allow us to probe the system close to the estimated pK_a value of the carboxylic acid, as well as observing what happens at the extremes (i.e. fully protonated at pH 2 and fully deprotonated at pH 7). Above pH 7, the high concentration of hydroxide ions in solution leads to the formation of a metathesis inactive $Ru-(OH)_n$ carbene complex, known to inhibit polymerisation.²⁴

As seen previously, an increase in turbidity was observed for all solutions. SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.2$) for each different pH solution (Table 2.2 and Figure S2.11).

Table 2.2. Characterisation summary of $P_{pH}1_{200}$.

Polymer	$M_{n,theo}$ / kDa	$M_{n,SEC}$ / kDa	$\mathcal{D}_{SEC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
$P_{pH3}1_{200}$	51.8	59.0	1.11	74	0.40	75 ± 13
$P_{pH4.5}1_{200}$	51.8	53.1	1.13	36	0.28	22 ± 1
$P_{pH7}1_{200}$	51.8	45.9	1.24	24	0.32	27 ± 1

The nano-objects formed at DP 200 were analysed by DLS, with average mean diameters of 82, 74, 36 and 24 nm for pH 2, 3, 4.5 and 7, respectively (Table 2.2 and Figure 2.10). The pH 3,

4.5 and 7 solution data showed a multimodal distribution, with a second peak at around 1000 nm. However, this size particle was not observed by TEM analysis, and so we attribute it to external contamination.

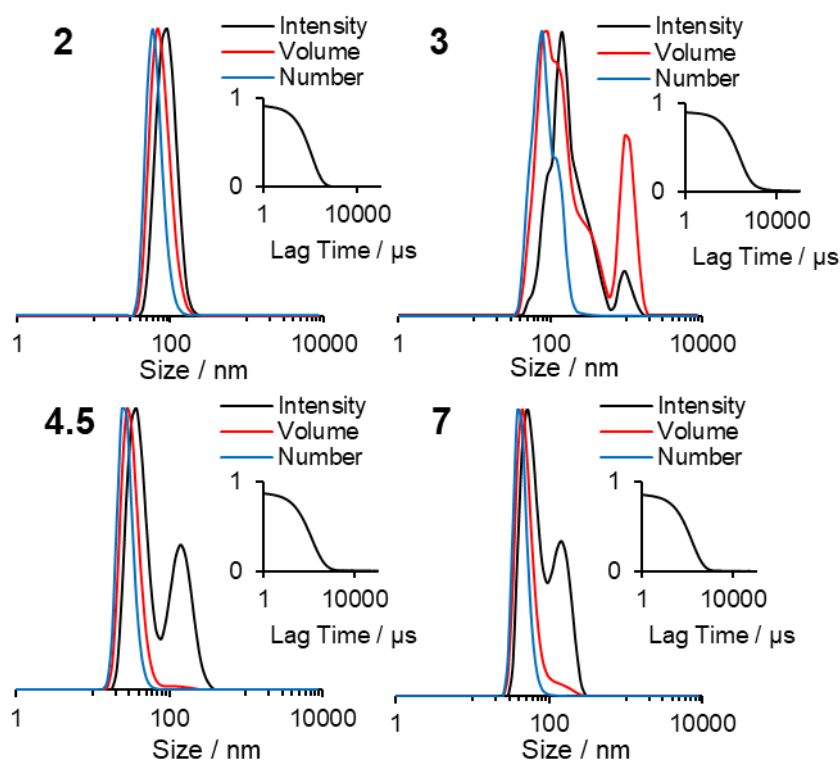


Figure 2.10. Normalised DLS analysis of $P_{pHy}1200$ particles synthesised at pH 2, 3, 4.5, and 7.

TEM analysis showed the presence of spherical nano-objects for ROMPISA solution above pH 4.5, but fused tubes at pH 3, as observed when synthesised at pH 2 (Table 2.2 and Figure 2.11, S2.12 and S2.13). The extent of fusion also appears to be pH dependent, with discrete, short tubes formed by successive fusion events at pH 3. However, at pH 2, the tubes form an interwoven network. This variance in fusion morphology may be due to the difference in degree of deprotonation within the carboxylic acid block at pH 2 compared to pH 3.

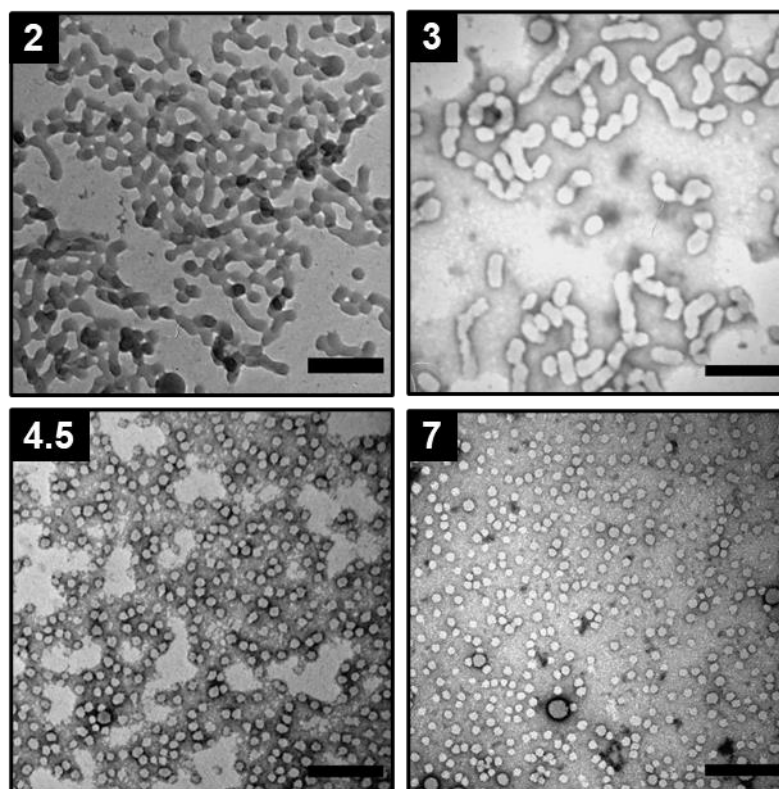
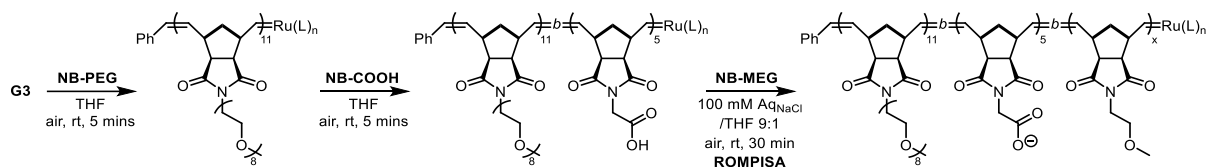


Figure 2.11. Dry-state TEM images of $P_{pHy1200}$ particles synthesised at pH 2, 3, 4.5, and 7. Scale bar = 200 nm.

2.3.1.3 Synthesis and characterisation of carboxylic acid-containing triblock nano-objects *via* ROMPISA in salt solution

The use of a phosphate buffer ensures solution pH remains constant throughout the preparation and analysis of carboxylic acid containing polymersomes. However, to switch the pH of the buffer solution a high concentration of acid is required to overcome the buffer. This may result in unwanted processes (e.g. side reactions or significant dilution) which could impact the self-assembled structures. Instead, synthesising carboxylic acid-containing triblock polymers, $P1_x$, without a buffer would allow for investigation into the effect of pH change on nano-object morphology at smaller intervals. A previous report from our group demonstrated that aqueous ROMPISA can be performed at neutral pH with the addition of various chloride

sources.²⁵ As such, **P1_x** synthesis was performed analogously as above but with the core forming monomer, **NB-MEG**, dissolved in a solution of 100mM NaCl in H₂O (Aq_{NaCl}) (Scheme 2.3).



Scheme 2.3. Synthetic route for the preparation of **P1_x** block copolymers by ROMPISA at pH 7.

As before, ¹H NMR spectroscopy was used to determine a > 99% conversion of **NB-MEG** within 30 minutes (Figure S2.14, S2.15 and S2.16). The solution at the end of the reaction was measured at pH 4.2. SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.4$) for all core DPs (Table 2.3 and Figure 2.12). Disparity in molecular weight (M_n) values between prediction and SEC analysis is likely attributed to the negative charge associated with the carboxylic acid group, which in turn lowers the efficacy of a PMMA standard for determining the molecular weight of the sample.²⁶

Table 2.3. Characterisation summary of **P1_x**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,SEC}$ / kDa	$\mathcal{D}_{SEC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm	Zeta Potential / mV
P1₁₀₀	29.7	22.6	1.34	54	0.27	18 ± 2	-48
P1₂₀₀	51.8	25.7	1.34	60	0.25	23 ± 1	-33
P1₃₀₀	73.9	26.6	1.36	71	0.24	24 ± 3	-33

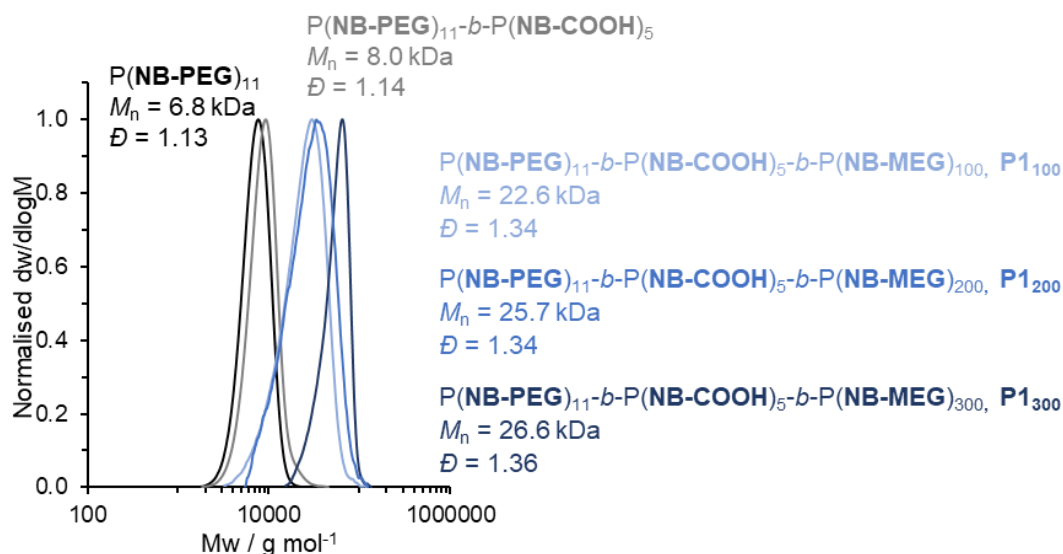


Figure 2.12. Normalised SEC traces (THF eluent, PMMA standards) of **P1_x** and intermediate polymers.

DLS analysis of the nano-objects formed showed a bimodal population, with peaks around 40 and 120 nm for all core DPs (Table 2.3 and Figure 2.13). The morphology for all samples was determined as spherical by TEM analysis with average diameters of 18, 23 and 24 nm for core DP 100, 200 and 300, respectively (Table 2.3 and Figure 2.14, S2.17 and S2.18).

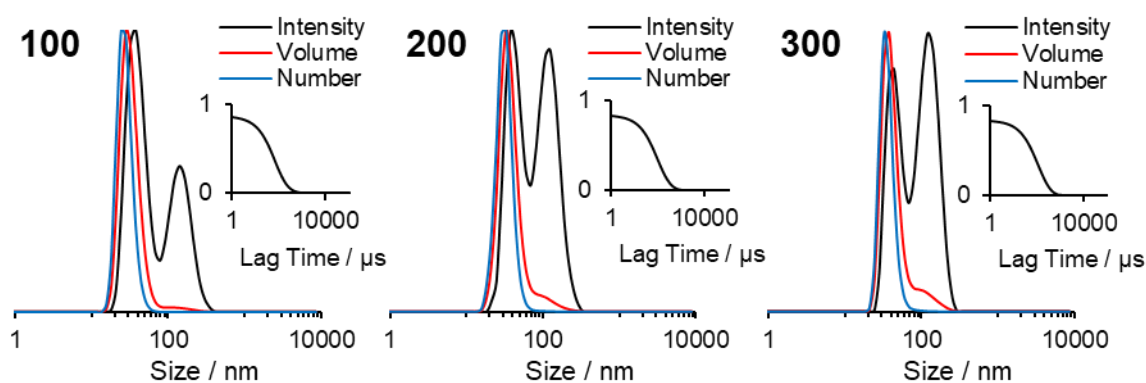


Figure 2.13. Normalised DLS analysis of **P1_x** particles: x = 100, 200, and 300.

Due to the slower polymerisation rate, some particles are able to undergo chain rearrangement during self-assembly to reduce membrane tension. This results in the formation of more thermodynamically favoured products with a larger overall diameter. In all

cases, zeta potential analysis confirmed the presence of negative charges on the outer surface of the obtained nano-objects (Table 2.3).

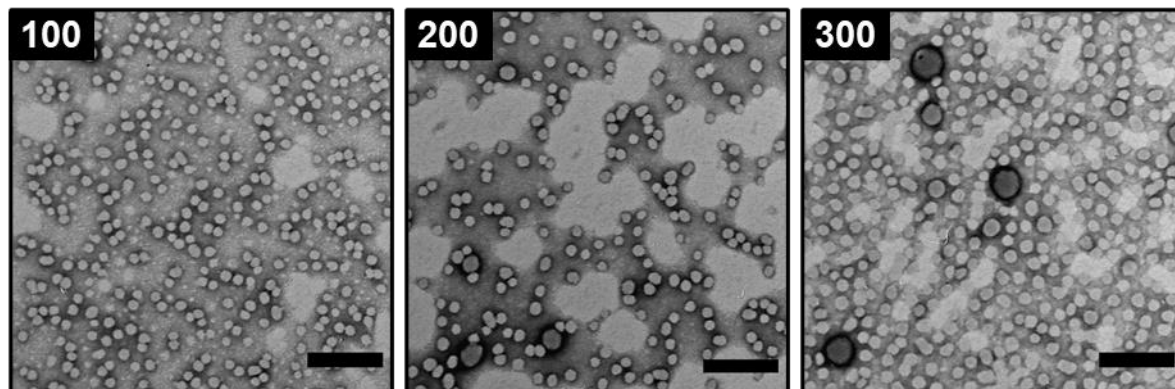


Figure 2.14. Dry-state TEM images of **P1_x** particles: $x = 100, 200$, and 300 . Scale bar = 200 nm .

2.3.1.4 Triggered polymersome fusion of carboxylic acid-containing triblock nano-objects

Preparing **P1₂₀₀** nano-objects at different pH demonstrated that COOH protonation state influences particle self-assembly, leading to a morphology shift from spherical nano-objects ($\text{pH} \geq 4$) to fused tubes ($\text{pH} \leq 3$). By switching the nano-objects from pH 7 to 2 and thus protonating the carboxylate groups, we hoped we could trigger fusion of the spherical particles to tubes.

It has already been shown that, below pH 3, the resulting morphology can still vary depending on the pH. Therefore, it was hypothesised that the resulting morphology following a pH trigger would be impacted by the concentration of acid added. As such, three volume equivalents of aqueous HCl solutions (+ 10 vol % THF) at different concentrations were added to **P1₂₀₀** particles. Samples were left overnight, during which an increase in turbidity was observed for concentrations above 10 mM HCl. Following this, the solutions were then analysed by DLS and dry-state TEM analysis to determine the resulting morphology (Table 2.4 and Figure 2.15 and S2.19).

Table 2.4. Characterisation summary of **P1₂₀₀** particles acidified with different concentrations of HCl.

HCl Concentration / mM	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
5	67	0.28	34 ± 2
10	67	0.25	43 ± 4
25	66	0.17	83 ± 11
50	78	0.11	88 ± 22
100	79	0.12	N/A ^a

^adry-state TEM image analysis was not possible due to the ambiguous start and end points of the fused particles.

TEM analysis showed no change in morphology upon addition of HCl below a concentration of 25 mM, with spherical nano-objects around 33 and 51 nm displayed for 5 and 10 mM HCl, respectively (Figure 2.15). Following the addition of the acid, the solution pH was measured between 4.2-4.6, which is unlikely to be acidic enough to protonate the carboxylate groups within the particles. As such, significant charge repulsion between the particles remains and maintains the high energy barrier to fusion.

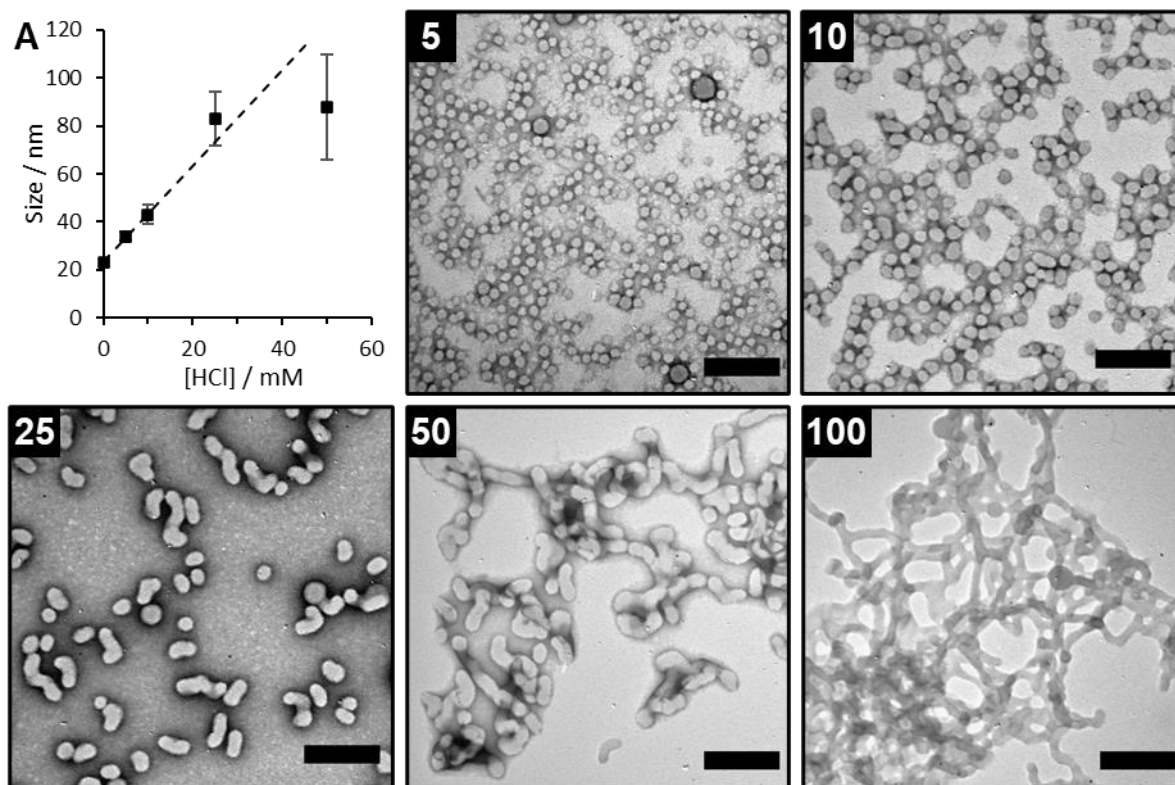


Figure 2.15. (A) Average size of particles acidified by different concentrations of acid, as determined by dry-state TEM analysis. Dry-state TEM images of $P1_{200}$ particles synthesised at pH 7 and acidified with different concentrations of HCl solution (+ 10% THF): 5, 10, 25, 50, and 100 mM. Scale bar = 200 nm.

In contrast, when a solution of 25 mM HCl or more concentrated was added to $P1_{200}$ particles, the resulting morphology showed fused tubes by TEM analysis, alongside an increase in mean diameter size by DLS. The higher concentration of acid allows for a greater proportion of carboxylate protonation and reduces the static repulsion between particles. This results in a lower energy barrier to fusion, of which the membrane tension within the particles can overcome and drive the fusion process (Figure 2.16). Longer, fused tubes are produced when a higher concentration of acid is used to switch the pH and trigger fusion. This may suggest that a higher concentration of acid leads to a greater degree of protonation and thus a lower electrostatic repulsion between particles. This results in a further reduced energy barrier to

fusion and, given membrane tension trapped within the particles is the same, results in an increased length of tube from subsequent fusion events (Figure 2.16).

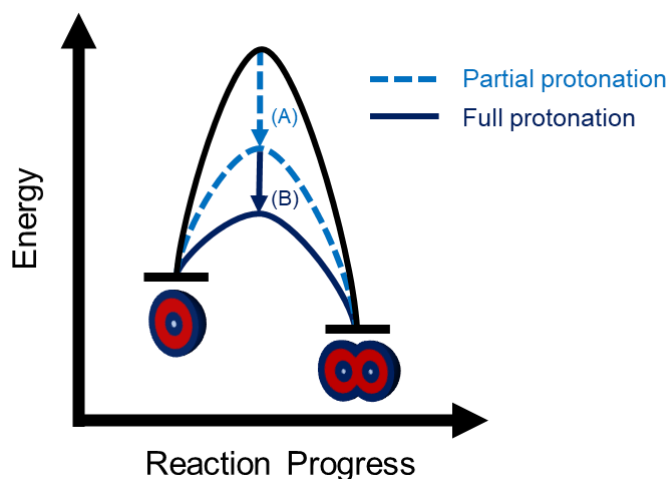


Figure 2.16. Hypothesised energy diagram of the first step in pH-triggered fusion of triblock nano-objects containing carboxylic acid groups that are either (A) partially protonated (dashed) or (B) fully protonated (solid).

Addition of 100 mM HCl led to the uncontrolled fusion of **P1₂₀₀** particles, forming an interconnected network of fused tubes which results in the gelation of the sample. This is likely due to the full protonation of carboxylic acid groups along the polymer chain and significantly reducing the energy barrier to particle fusion. TEM analysis to assess particle length was possible with 50 mM but not 100 mM and so an acid concentration of 50 mM HCl was selected for future triggered fusion experiments.

Following this, to determine the DP dependence on triggered fusion of carboxylic acid-containing polymersomes, three volume equivalents of 50mM HCl solution (+ 10 vol % THF) was added to **P1_x** particles to switch the pH from 7 to 2, forming a final solution of 3:1 HCl solution to particle solution. No change in turbidity was observed during the first 30 minutes,

in contrast to only 5 minutes seen in their amine-functionalised equivalent.²⁰ Samples were then left overnight during which an increase in turbidity was observed for **P1₂₀₀** and **P1₃₀₀**. DLS analysis showed an increase of 27, 54 and 137 nm in average diameter for DP 100, 200 and 300, respectively, whilst fusion from spherical nano-objects to anisotropic structures was observed for core DPs ≥ 200 by TEM (Table 2.5 and Figure 2.17, 2.18, 2.19, S2.20 and S2.21).

Table 2.5. Characterisation summary of **P1_x** particles acidified to pH 2.

Polymer	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
P1₁₀₀ @ pH 2	81	0.60	16 ± 2
P1₂₀₀ @ pH 2	114	0.57	85 ± 15
P1₃₀₀ @ pH 2	208	0.56	99 ± 20

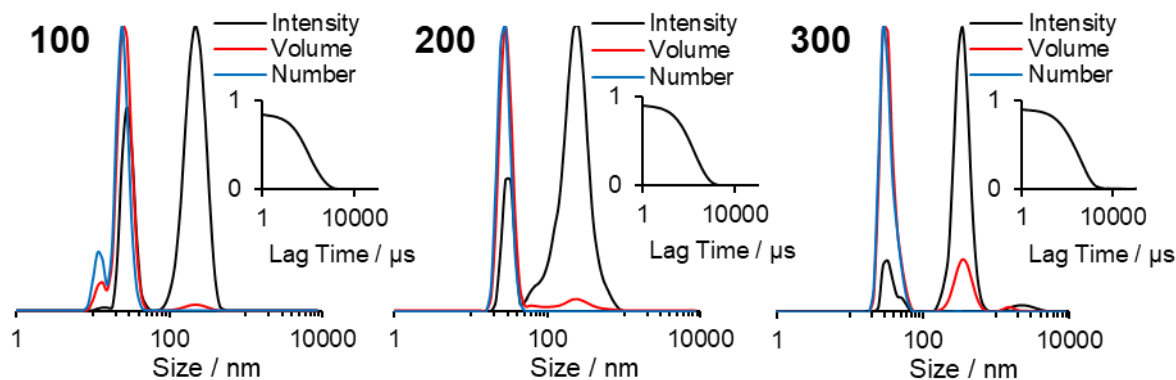


Figure 2.17. Normalised DLS analysis of **P1_x** particles acidified to pH 2: $x = 100, 200$, and 300 .

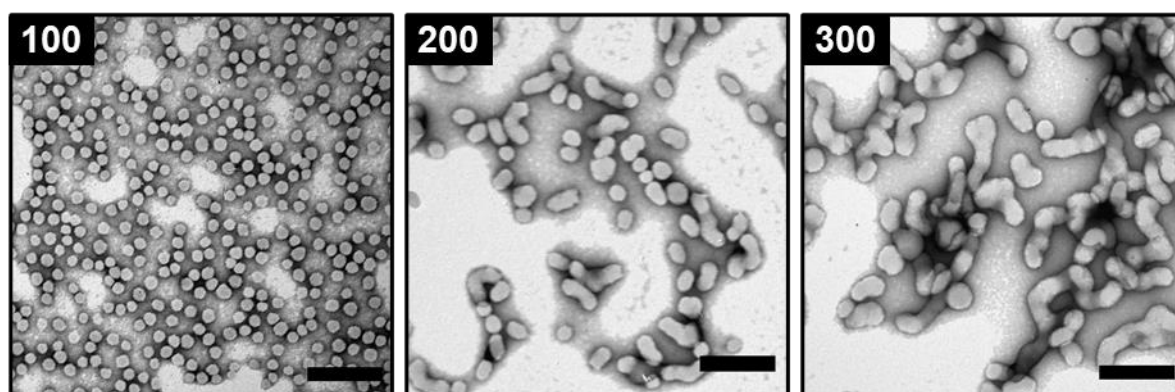


Figure 2.18. Dry-state TEM images of $P1_x$ particles synthesised at pH 7 and acidified to pH 2: $x = 100$, 200, and 300. Scale bar = 200 nm.

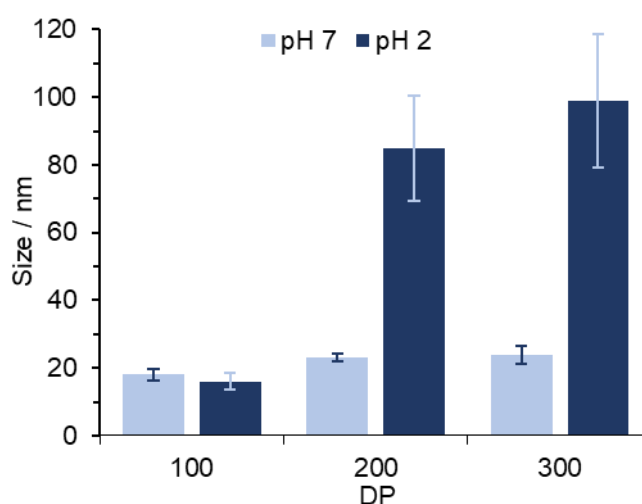


Figure 2.19. Average size of $P1_x$ particles synthesised at pH 7 (light blue) and acidified to pH 2 (dark blue), as determined by dry-state TEM.

Carboxylic acid-functionalised polymersomes that were synthesised at pH 7 and acidified to pH 2 (Figure 2.18) formed shorter fused structures compared to nano-objects formed at pH 2 (Figure 2.9), when core DP is consistent. At pH 7, the rate of polymerisation is slower than at pH 2, allowing more time for chain rearrangement to occurring during self-assembly. This results in polymersomes with lower membrane tension and thus a lower thermodynamic driving force for fusion. This shows a clear pathway dependence in polymersome fusion, with

polymerisation rate and the resulting membrane tension attributed as a key factor in determining the degree of fusion.

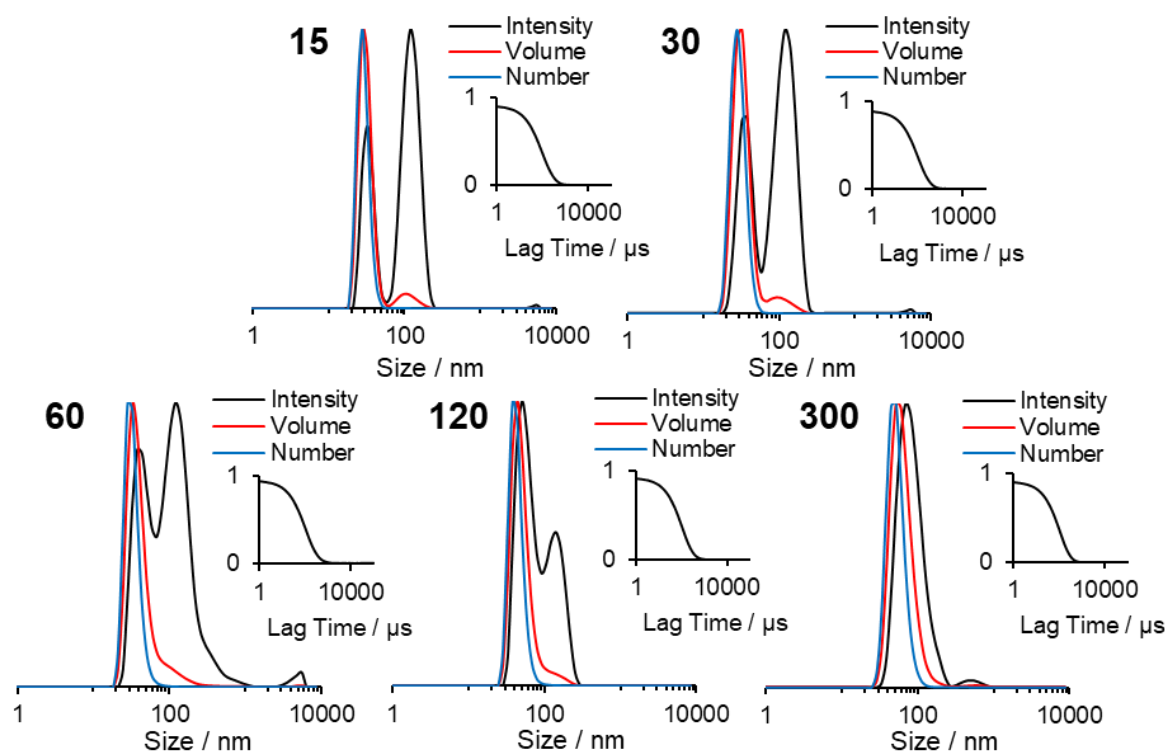
However, the tube length of triggered **P1_x** is far greater than previously reported amine-functionalised tubes.²⁰ For **P1₂₀₀**, there is an average increase of 370% in particle length upon triggered fusion - much higher than the 175% increase seen in analogous amine-containing particles. This may be due to a reduction in branching during successive fusion events, resulting in long, linear tubes. Alternatively, previously calculated surface area-normalised octanol/water partition coefficients ($\text{LogP}_{\text{oct}}/\text{SA}$) indicate that carboxylic acid-containing monomers are very hydrophobic when protonated (i.e. at low pH). This large shift in hydrophobicity upon protonation acts as a driving force for fusion, resulting in longer tubes being formed.

2.3.1.5 Mechanism of triggered polymersome fusion of carboxylic acid-containing triblock nano-objects

When triggered by addition of base, amine-functionalised pH-responsive polymersomes undergo rapid fusion, with the full process complete within a couple of minutes.²⁰ In contrast, no change in solution turbidity was observed for carboxylic acid polymersomes in the same time period. To ascertain the timescale of triggered fusion, **P1₂₀₀** particles were acidified by addition of HCl with aliquots of the acidified solution taken at regular intervals and analysed by DLS and TEM (Table 2.6 and Figure 2.20 and 2.21).

Table 2.6. Characterisation summary of **P1_x** particles acidified to pH 2 at different time points.

Time / min	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
15	70	0.31	19 ± 2
30	69	0.30	28 ± 3
60	76	0.34	30 ± 4
120	71	0.22	37 ± 5
300	74	0.16	75 ± 14

**Figure 2.20.** Normalised DLS analysis of **P1₂₀₀** particles synthesised at pH 7 and acidified to pH 2, analysed at different time points: 15, 30, 60, 120, and 300 minutes.

DLS analysis showed a consistent average hydrodynamic diameter as the reaction progressed, with a shift from a bi-modal to mono-modal distribution after 300 minutes (Figure 2.20). TEM analysis showed predominantly spherical nano-objects between 0-30 minutes followed by the

formation of anisotropic structures at 60 minutes, which continue to grow in length over the next 4 hours (Figure 2.21 and S2.22).

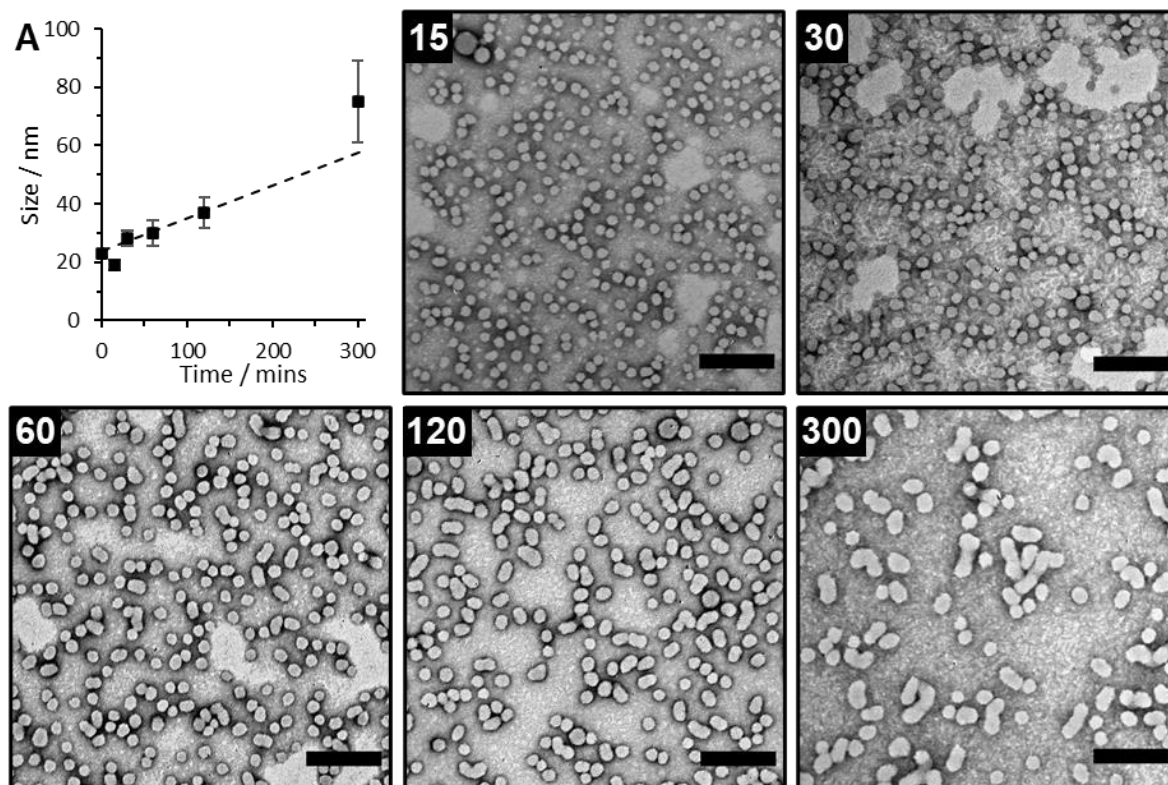


Figure 2.21. (A) Average size of particles synthesised at pH 7 and acidified to pH 2, analysed at different time points, as determined by dry-state TEM analysis. Dry-state TEM images of **P1₂₀₀** particles synthesised at pH 7 and acidified to pH 2, analysed at different time points: 15, 30, 60, 120, and 300 minutes. Scale bar = 200 nm.

Previous work in this area, has shown that triggered polymersome fusion occurs over two steps: (1) particles first adhere to one another to give elongated particles; (2) these then undergo polymer chain rearrangement to give the final fused product (Figure 2.22). In amine containing particles, the rate-determining step of fusion was ascertained to be structural rearrangement, supported by the observation of adhered but not fully fused structures in TEM analysis. In addition, the length of particles was shown to evolve more quickly than the width

by *in situ* SAXS analysis.²⁰ As such, the activation energy for chain rearrangement (E_{a2}) must be greater than the activation energy for adhesion (E_{a1}) (Figure 2.22).

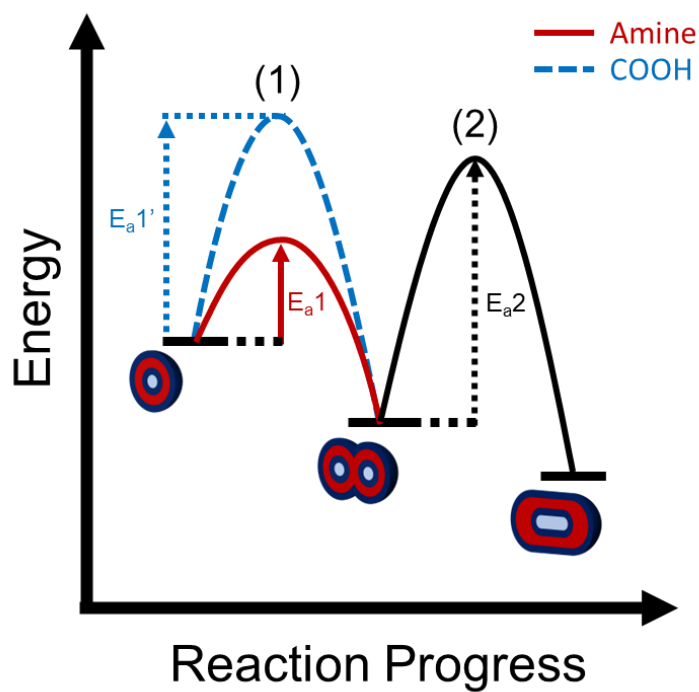


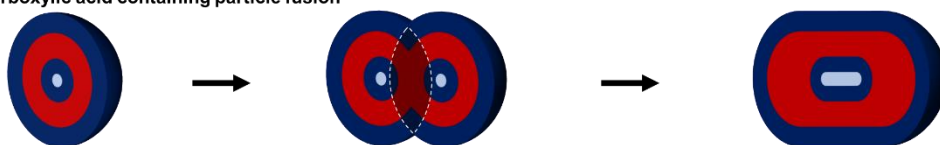
Figure 2.22. Hypothesised energy diagram for the two-step pH-triggered fusion of triblock nano-objects containing either amine (red solid) or carboxylic acid groups (blue dashed).

Because the polymers discussed in that study only differ from **P1₂₀₀** by five units (2 % of total number of targeted units), we assumed the activation energy for chain rearrangement (E_{a2}) would be similar for both particles, with the **P(NB-MEG)₂₀₀** block dominating the thermodynamics of the process. If this step is the rate-determining step for **P1₂₀₀** fusion, the overall rate of fusion should be very similar to that for amine-containing triblock nano-objects. However, the overall rate is slower for carboxylic-acid containing nano-objects. From this, we can deduce that the rate-determining step for **P1₂₀₀** fusion is the initial adhesion of particles (1). This is supported by intermediate structures not being observed by TEM (Figure 2.21). If the rate-determining step for fusion of carboxylic acid containing nano-objects is particle

adhesion, the activation energy for this step ($E_{a1'}$) must be greater than that of structural rearrangement (E_{a2}) (Figure 2.22).

Carboxylic acid containing particles are smaller than their amine equivalent (23 and 36 nm by dry-state TEM, respectively), meaning they possess a higher membrane curvature. It is known that, for fusion of polymersomes to occur, the cores of the two membranes must come into contact, creating a hemi-fused state.^{27, 28} In smaller particles, in this case **P1₂₀₀**, the extent of steric repulsion between polymer chains during fusion is larger due to the greater proportion of polymer chains overlapping in order to bring the cores into contact (Figure 2.23). This repulsion leads to a higher activation energy for the adhesion step and results in a switch of the rate-determining step of fusion.

A. Carboxylic acid containing particle fusion



B. Amine containing particle fusion

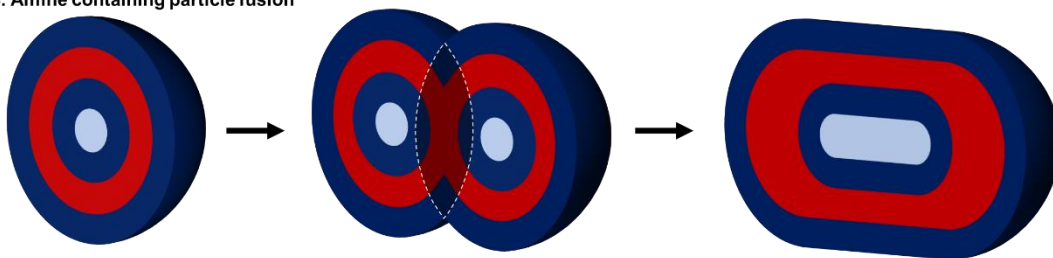


Figure 2.23. Schematic showing the two-step fusion process and their coronal overlap for triblock nano-objects of different sizes.

This theory is further supported by comparing the morphology of the resulting fused product for carboxylic acid and amine containing triblock nano-objects. In amine-functionalised polymersomes, the slow rate of structural rearrangement resulted in the formation of

branched fusion products. Due to the disparity in rate of the two steps, branches were often formed, likely due to the adhesion of a third polymersome in an orthogonal direction prior to the completion of the chain rearrangement step, initiating a second fusion sequence (Figure 2.24, A).

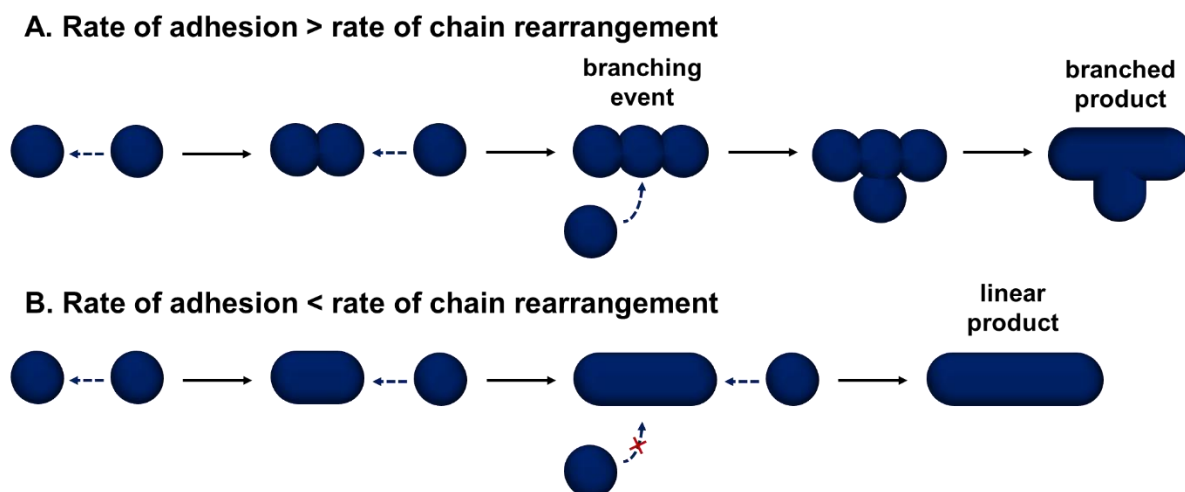


Figure 2.24. Suggested fusion mechanism when the rate of adhesion is (A) greater than or (B) less than the rate of chain rearrangement.

In contrast, upon pH switch, **P1₂₀₀** only display fusion products resulting from combination of polymersomes in a unilateral direction. This supports the theory that the adhesion of polymersomes is the rate-determining step in this case and prevents the formation of a secondary fusion sequence occurring in a disparate direction (Figure 2.24, B). This process could be likened to a nanoscopic version of the Hammond Postulate, which states that the transition state of a reaction resembles the structure of the species (reactant or product) to which it is closer in energy.²⁹ That is to say, the higher activation energy for adhesion of carboxylic acid-functionalised polymersomes, compared to those containing amine groups, increases the selectivity of the fusion process, resulting in linear tubes, rather than branched structures.

2.3.2 Dual pH-responsive particles *via* ROMPISA

Previous work has shown that the release of membrane tension triggered by pH switch can be achieved over multiple steps by incorporating basic monomers with differing pK_a values.²⁰ However, this was achieved in one direction, i.e. pH was increased initially to 7 and then further to pH 12. With the development of carboxylic acid-functionalised polymersomes that respond to a decrease in pH, it was theorised that both amine- and carboxylic acid-functionalised monomers could be utilised within a polymer chain (Figure 2.25). This would allow access to zwitterionic polymersomes that are dual pH-responsive and can be triggered to fuse by either an increase or decrease in solution pH.

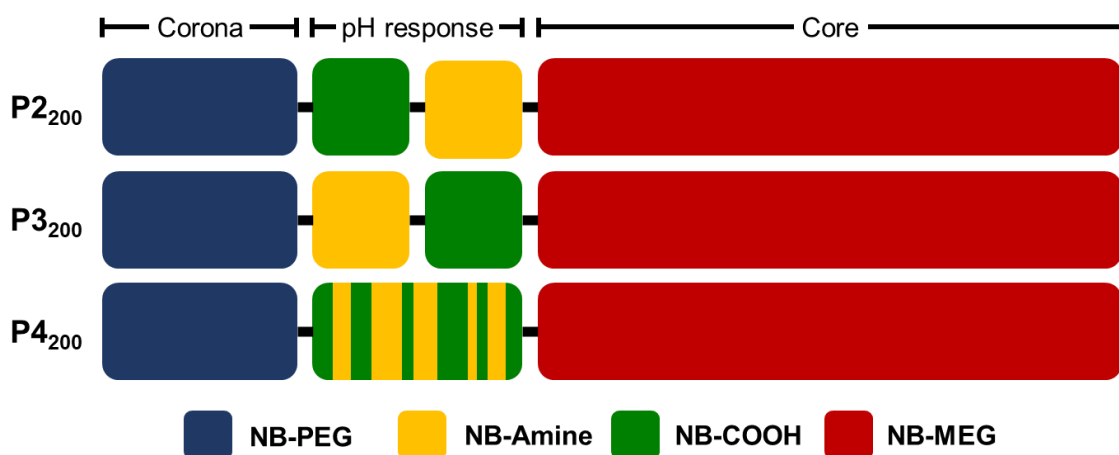


Figure 2.25. Schematic diagram showing the different block orders and microstructures targeted for the polymers in each dual pH-responsive particle.

In addition to this, the ability of these polymersomes to undergo dual, stepped pH-responsive fusion behaviour, following a switch between basic (i.e. pH 12) and acidic (i.e. pH 2) conditions, and whether there was any pathway dependence to this process (i.e. does the order of stimuli matter) was investigated.

Finally, polymer microstructure has already been shown to have an impact on the products formed during ROMPISA polymersome fusion.²⁰ As such, the impact of block order, as well as microstructure (block copolymer vs. random copolymer), on the fusion of dual pH-responsive zwitterionic polymersomes will be investigated (Figure 2.25).

2.3.2.1 Synthesis and characterisation of zwitterionic nano-objects *via* ROMPISA at pH 7

To prepare dual pH-responsive zwitterionic polymersomes, three amphiphilic block copolymers were synthesised *via* ROMPISA (Scheme S2.1). Firstly, P(**NB-PEG**)₁₁-*b*-P(**NB-COOH**)_{2.5}-*b*-P(**NB-Amine**)_{2.5}P(**NB-MEG**)₂₀₀, **P2**₂₀₀, containing a corona formed from discrete blocks of **NB-PEG**, **NB-COOH** and **NB-Amine**, and a core of **NB-MEG** (DP = 200). Secondly, the pH-responsive blocks were inverted to form P(**NB-PEG**)₁₁-*b*-P(**NB-Amine**)_{2.5}-*b*-P(**NB-COOH**)_{2.5}-*b*-P(**NB-MEG**)₂₀₀, **P3**₂₀₀. In addition, to investigate the impact of microstructure on fusion, an analogous polymer, P(**NB-PEG**)₁₁-*b*-(P(**NB-COOH**)_{2.5}-*r*-P(**NB-Amine**)_{2.5})-*b*-P(**NB-MEG**)₂₀₀, **P4**₂₀₀, containing a random copolymer block of **NB-COOH** and **NB-Amine**, was synthesised.

The syntheses were performed analogously to a previously reported method of quadrablock polymersome synthesis.²⁰ For **P2**₂₀₀, the corona was synthesised by reacting the **G3** initiator with the hydrophilic monomers, first **NB-PEG**, then **NB-COOH** and finally **NB-Amine** in tetrahydrofuran (THF). The resulting water-soluble macroinitiator was then chain-extended upon addition of **NB-MEG** dissolved in 100 mM Aq_{NaCl} to give a final solvent composition of THF/Aq_{NaCl} of 1:9 v/v. This process was then repeated but with **NB-Amine** being added before **NB-COOH** to produce **P3**₂₀₀. Lastly, **P4**₂₀₀ was synthesised by adding both **NB-COOH** and **NB-Amine** together to produce a random copolymer block. The properties of all zwitterionic particles synthesised are detailed in Table S2.2.

An increase in turbidity was observed for all solutions. SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.2$) for all polymers (Table 2.7 and S2.3 and Figure 2.26, 2.27 and 2.28).

Table 2.7. Characterisation summary of **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,SEC}$ / kDa	$\mathcal{D}_{SEC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm	Zeta Potential / mV
P2₂₀₀	51.8	44.3	1.29	57	0.26	33 ± 2	-24
P3₂₀₀	51.8	37.1	1.49	57	0.30	33 ± 2	+21
P4₂₀₀	51.8	41.8	1.39	56	0.25	36 ± 2	+0.8

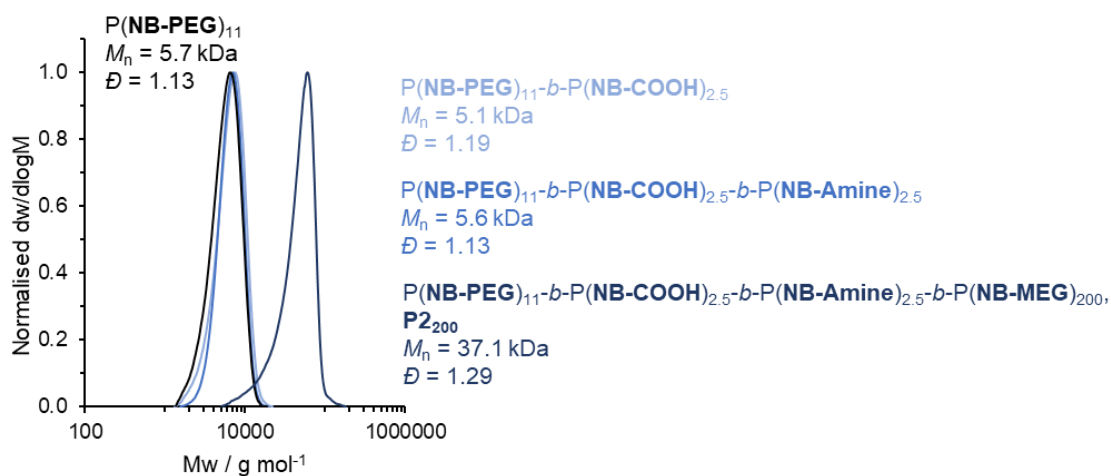


Figure 2.26. Normalised SEC traces (THF eluent, PMMA standards) of **P2₂₀₀** and intermediate polymers.

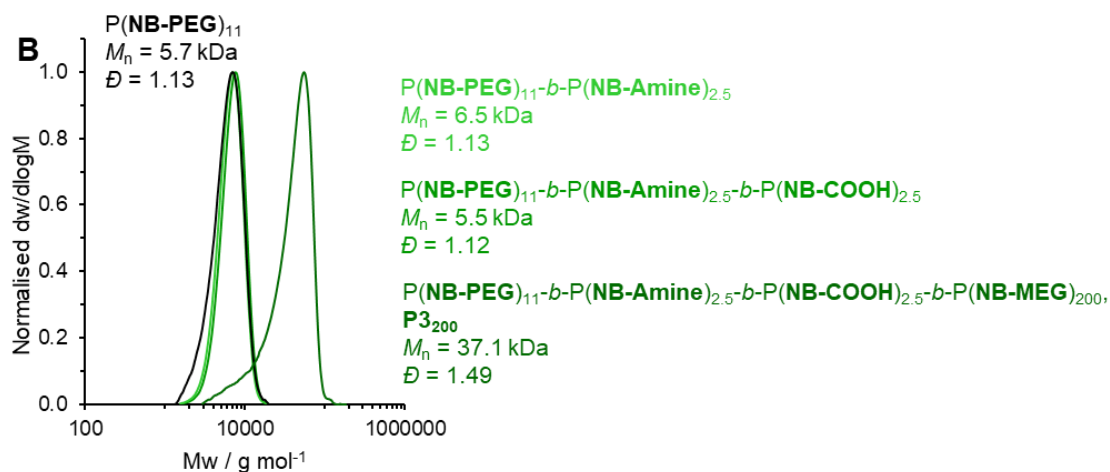


Figure 2.27. Normalised SEC traces (THF eluent, PMMA standards) of **P3₂₀₀** and intermediate polymers.

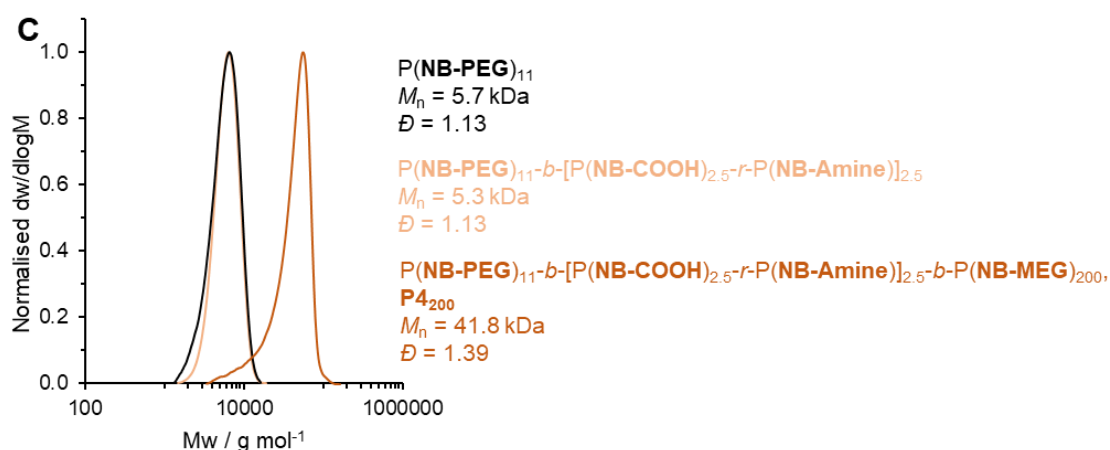


Figure 2.28. Normalised SEC traces (THF eluent, PMMA standards) of **P4₂₀₀** and intermediate polymers.

DLS analysis of the nano-objects formed showed a bimodal population, with a main peak around 50 nm and a small, secondary peak above 100 nm for all polymers (Table 2.7 and Figure 2.29). The morphology for all samples was determined as spherical, although a small amount of anisotropy was seen in all samples, by TEM analysis with average diameters of 33, 33 and 36 nm for **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀**, respectively (Table 2.7 and Figure 2.30, S2.23 and S2.24). These results indicate no dependence of either block order or microstructure on the synthesis of particles at pH 7. Zeta potential analysis indicated that **P2₂₀₀** particles were anionic and **P3₂₀₀** cationic, suggesting the pH-responsive block closest to the corona block effectively screens

the third block, whilst **P4₂₀₀** particles were determined to be uncharged (Table 2.7 and Figure S2.25).

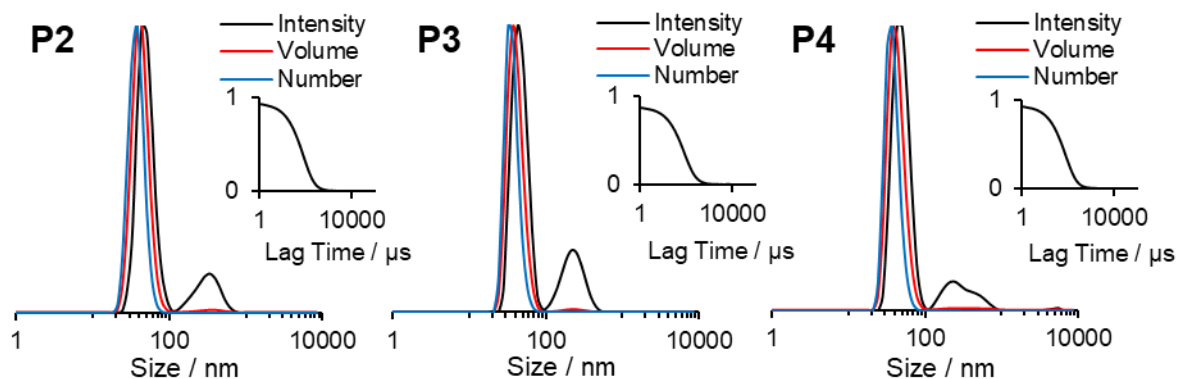


Figure 2.29. Normalised DLS analysis of **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀**.

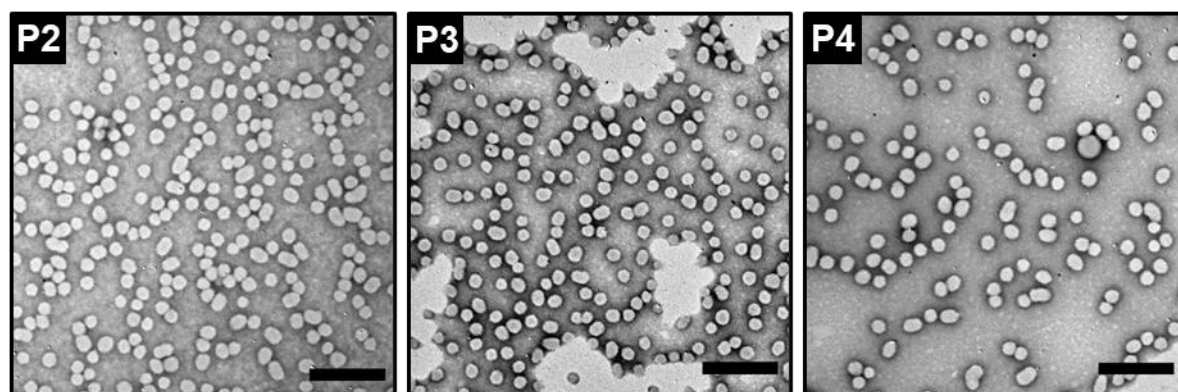


Figure 2.30. Dry-state TEM images of **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀**. Scale bar = 200 nm.

2.3.2.2 Dual pH triggering of zwitterionic nano-objects

To ascertain the ability of the zwitterionic nano-objects to undergo dual pH-triggered fusion, the pH of each polymer solution (**P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀**) was first increased to pH 12 by addition of base, followed by a switch to pH 2. This was also repeated in the reverse order: the pH was first lowered to pH 2 and then switched to 12. The particle solutions were characterised by DLS and TEM at each step to determine the impact on particle morphology and size.

DLS analysis of the nano-objects acidified to pH 2 showed highly disperse ($PDI_{DLS} \geq 0.4$) multimodal distribution for each polymer solution, with the smallest peak around 50 nm, in line with the original particles synthesised at pH 7. In addition to this, there are several peaks above 100 nm, most likely due to the aggregation of particles or other salts present in the solution (Table 2.8 and Figure S2.26).

Table 2.8. Characterisation summary of **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀** acidified to pH 2 then switched pH 12.

Polymer	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
P2₂₀₀ @ pH 2	108	0.47	52 ± 9
P3₂₀₀ @ pH 2	194	0.53	38 ± 4
P4₂₀₀ @ pH 2	169	0.43	64 ± 7
P2₂₀₀ @ pH 2 → 12	113	0.45	67 ± 14
P3₂₀₀ @ pH 2 → 12	135	0.53	53 ± 10
P4₂₀₀ @ pH 2 → 12	135	0.53	94 ± 14

TEM analysis for the acidified particles showed an overall increase in average size for all samples, with particles measuring at 52, 38 and 64 nm for **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀**, respectively (Table 2.8 and Figure 2.31 and S2.27). A small number of fusion events were observed for **P2₂₀₀** and **P4₂₀₀** samples, suggesting that the hydrophobicity of the **NB-COOH** units at pH 2 is greater than the hydrophilicity of amine-functionalised units, allowing the entirety of the pH-responsive block to be buried within the membrane core, and promote fusion. However, the presence of positively charged amine groups within the membrane core results in

interspersed hydrophobic and hydrophilic regions which leads to the formation of irregularly fused structures, similar to “patchy” particles previously reported.^{20, 30, 31}

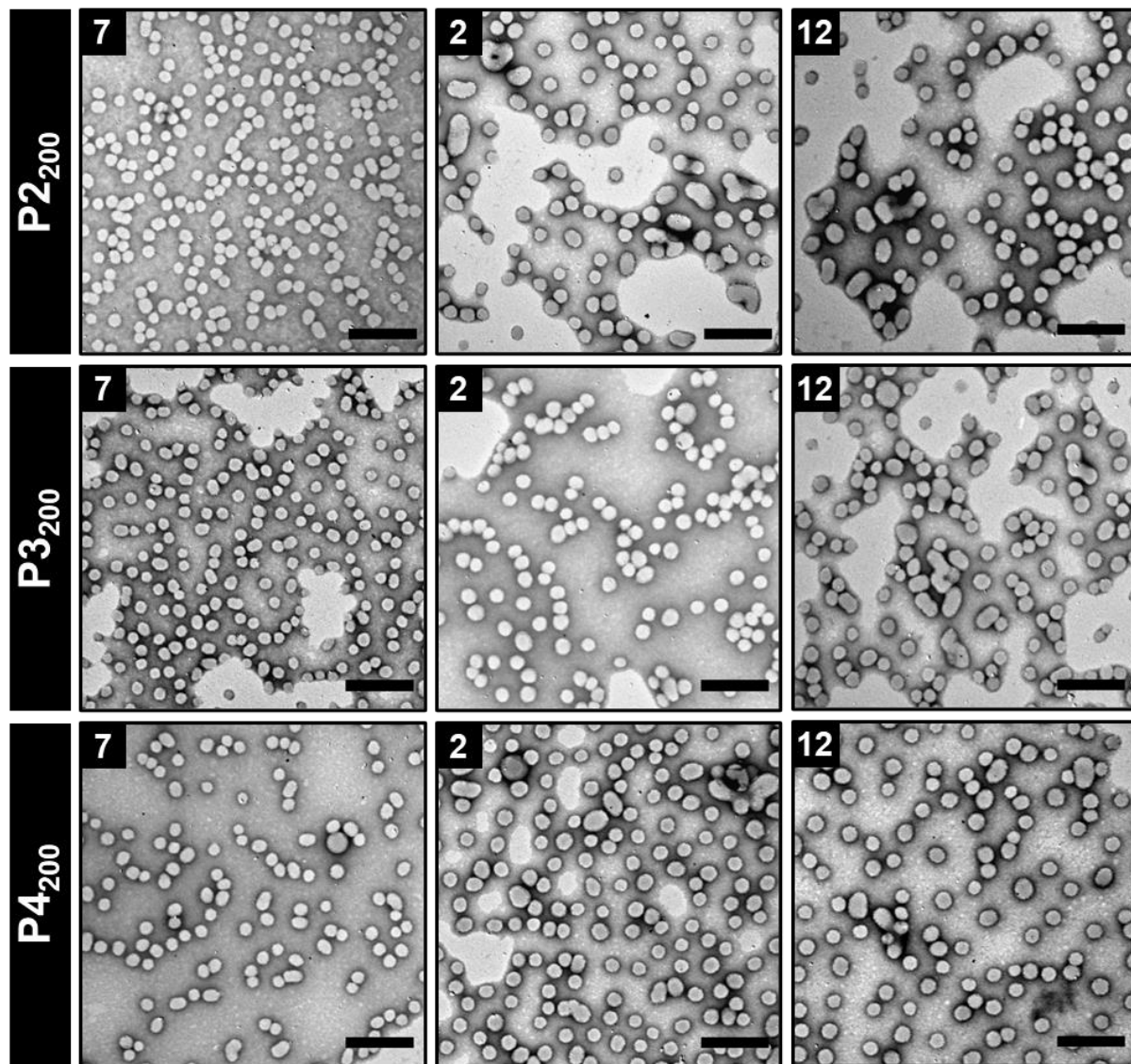


Figure 2.31. Dry-state TEM images of **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀** particles synthesised at pH 7, acidified to pH 2 and then basified to pH 12. Scale bar = 200 nm.

In contrast, **P3₂₀₀** particles showed a slight increase in average size of 5 nm whilst maintaining their spherical morphology (Figure 2.31 and S2.27). At pH 2, the carboxylic acid and amine groups are both protonated, resulting in the **NB-COOH** block switching to hydrophobic. As this block is closest to the core-forming block, it can easily embed itself within the core, whilst the

NB-Amine block remains as part of the hydrophilic corona. As such, the change in the hydrophilic/hydrophobic ratio is not significant enough to overcome the electrostatic repulsion between the positively charged amine groups, impeding the formation of fused structures.

Following this, DLS analysis for particles switched from pH 2 to 12 showed no change, with multimodal distributions for all 3 samples, which could be due to the formation of large salt aggregates (Table 2.9 and Figure S2.26).

Table 2.9. Characterisation summary of **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀** basified to pH 12 then switched pH 2.

Polymer	$Z_{\text{avg,DLS}}$ /nm	PDI_{DLS}	L_{TEM} /nm
P2₂₀₀ @ pH 12	54	0.12	56 ± 6
P3₂₀₀ @ pH 12	83	0.19	49 ± 6
P4₂₀₀ @ pH 12	55	0.15	60 ± 7
P2₂₀₀ @ pH 12 → 2	224	0.65	73 ± 14
P3₂₀₀ @ pH 12 → 2	219	0.76	87 ± 22
P4₂₀₀ @ pH 12 → 2	187	0.55	89 ± 21

TEM analysis of **P2₂₀₀** and **P4₂₀₀** samples at pH 12 show a further increase in average particle size of 15 and 30 nm, respectively but show no significant change in morphology (Table 2.9 and Figure 2.31 and S2.27). This further supports our suggestion that the entire pH-responsive block has been embedded within the core following the initial acidification step. The deprotonation of the carboxylic acid groups causes an increase in the electrostatic repulsion

between the chains, resulting in a “swelling” of the particles and an increase in average particle size.

By comparison, the morphology of **P3₂₀₀** particles changed significantly when switched from pH 2 to 12, with the formation of irregular fused structures observed (Figure 2.31 and S2.27). The increase in pH leads to the deprotonation of the P(**NB-Amine**) block, thus switching it to hydrophobic. This destabilises the particle structure and leads to the release of built-up membrane tension *via* particle fusion.

When the direction of pH switching is reversed, DLS analysis of zwitterionic nano-objects synthesised at pH 7 and basified to pH 12 displayed unimodal distributions for each polymer solution, with an average mean diameter of 54 and 55 nm for **P2₂₀₀** and **P4₂₀₀**, respectively, in line with the original particles synthesised at pH 7. In contrast, **P3₂₀₀** showed an average diameter of 83 nm, suggesting fusion may have occurred (Figure S2.28).

However, dry-state TEM analysis gave average size values between 50-60 nm and indicated fusion events had occurred for all 3 samples, suggesting neither block order nor microstructure have an influence on base triggered zwitterionic polymersome fusion (Figure 2.32 and S2.29). The samples underwent a small degree of fusion, with typically only 2-3 particles fusing to form a single tube. However, the percentage of anisotropic particles in the samples was much higher than that seen in the acidified samples (Figure 2.32 and S2.29). This would suggest there is significant membrane tension within the particles to fuse a couple of times but sequential fusion beyond this is prohibited.

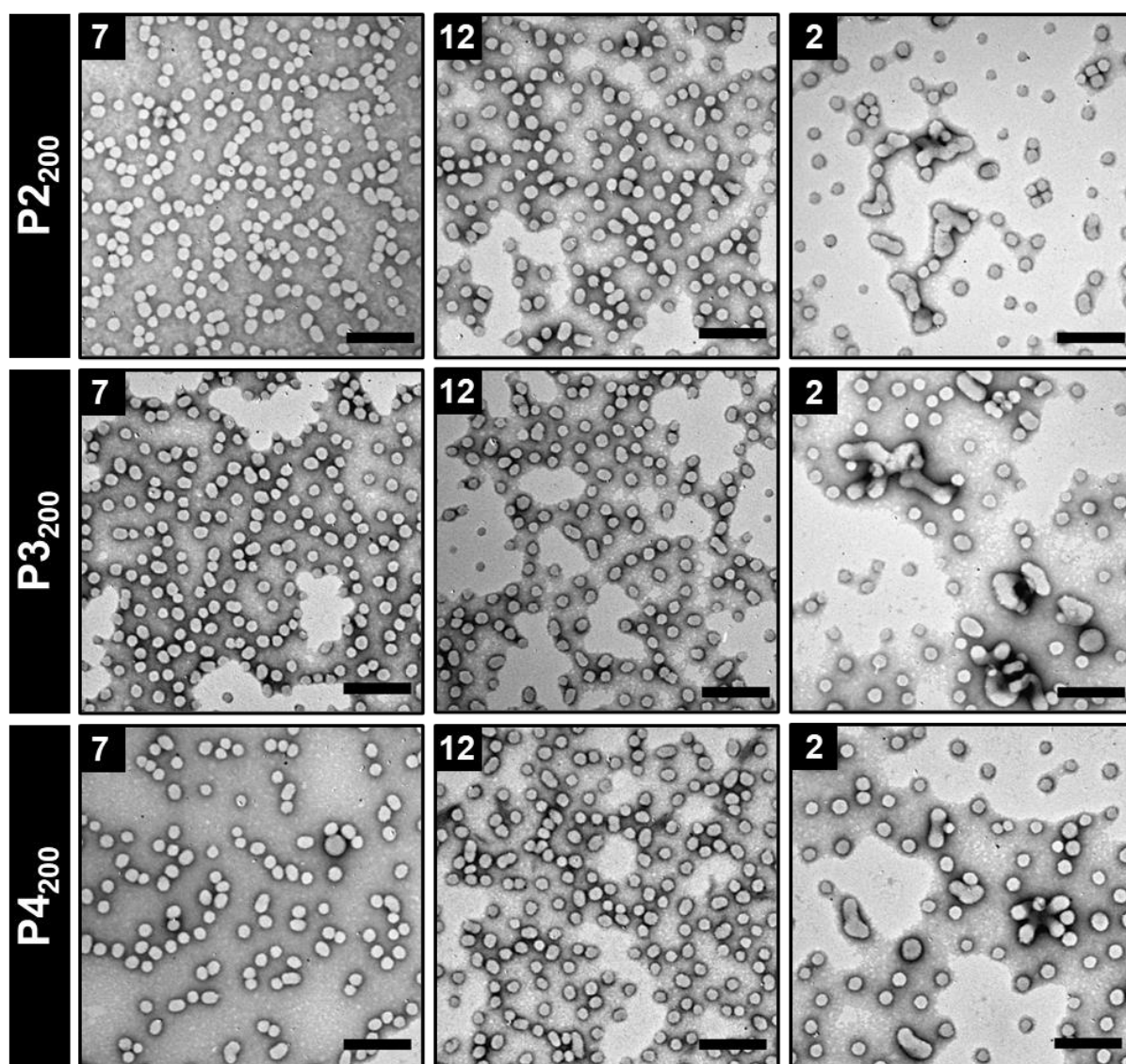


Figure 2.32. Dry-state TEM images of **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀** particles synthesised at pH 7, basified to pH 12 and then acidified to pH 2. Scale bar = 200 nm.

In addition, the smallest size increase (16 nm) was observed for **P3₂₀₀** particles which suggests the charged carboxylic acid block between P(**NB-Amine**) and the core prevents clean insertion of the hydrophobic amine block into the membrane, restricting the fusion process (Figure 2.33). In contrast the amine group in **P2₂₀₀** can freely transfer into the membrane, permitting a greater increase in average particle size (23 nm) (Figure 2.33).

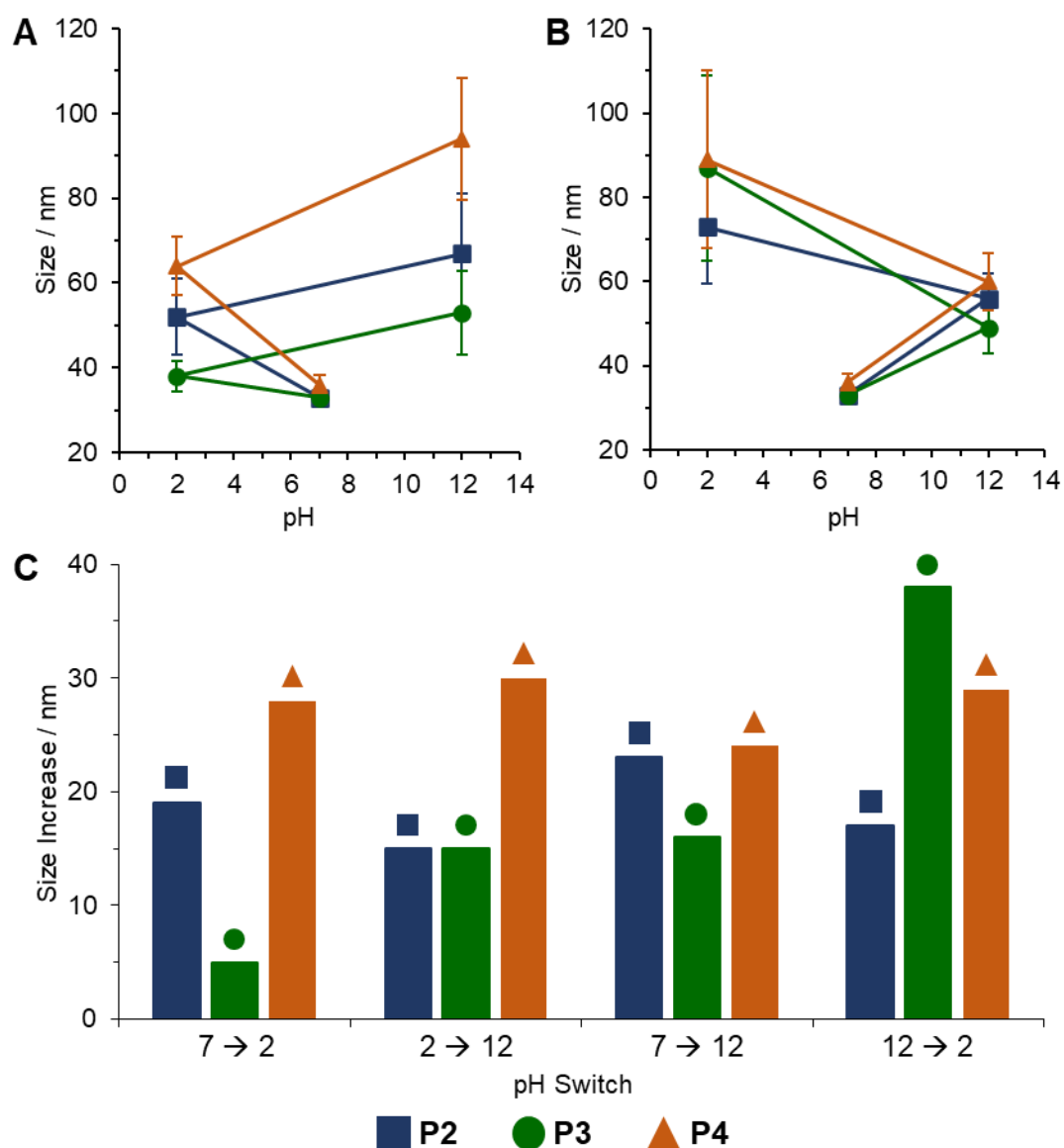


Figure 2.33. Average particle size, as determined by dry-state TEM, for **P2₂₀₀** (blue squares), **P3₂₀₀** (green circles), and **P4₂₀₀** (orange triangles) particles switched to (A) pH 2 then 12 and (B) pH 12 then 2. (C) Increase in particle size for each pH switch step for **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀** particles.

Moreover, DLS analysis for particles switched from pH 12 to pH 2 showed multimodal distributions for all 3 samples (Figure S2.28). This is similar to that seen for particles switched from pH 2 to pH 12. Further analysis by dry-state TEM again showed similar morphologies for all three particles, with a mixture of spherical particles and large irregular fused structures

present (Figure 2.32 and S2.29). As such, no dependence on block order or microstructure was observed in zwitterionic particles when the pH was switched to 12 and then 2.

All 3 samples showed an increase in average particle size during each pH switch (Figure 2.33). **P2₂₀₀** and **P4₂₀₀** both displayed similar particle size increases (19 and 28 nm, respectively), regardless of whether there was an increase or decrease in solution pH (Figure 2.33, blue squares and orange triangles). The greater size increase of **P4₂₀₀** compared to **P2₂₀₀** is likely due to the random distribution of amine- and carboxylic acid-functionalised monomers throughout the pH-responsive block, resulting in a shielding effect of any charge between the two groups. This reduces the electrostatic repulsion between particles and thus permits a higher degree of fusion to occur. This is further supported by **P2₂₀₀** particles having a greater particle increase during the first step, whilst **P4₂₀₀** increases particle size to the greatest extent on the second pH switch. Both **P2₂₀₀** and **P4₂₀₀** formed fused particles of a similar length, irrespective of pathway when dual pH-triggered across two steps.

In contrast, **P3₂₀₀** displayed a range of particles size increases (Figure 2.33, green circles). Particle size increase was consistent when switching to basic pH, irrespective of the starting pH (15 and 16 nm when switching from pH 2 and 7, respectively). This may be due to the entirety of the pH-responsive block becoming embedded within the core of the particle, causing a destabilisation of the hydrophilic/hydrophobic balance, and resulting in energy release *via* fusion. However, due to the deprotonation of the carboxylic acid groups at pH 12, electrostatic repulsion between particles is still high and thus restricts fusion to only 2-3 particles.

When switched from neutral to pH 2, no change in morphology was observed for **P3₂₀₀** and the average particle size increased by 5 nm. In contrast, when the pH of the particle solution is switched to pH 2 from pH 12, the greatest average particle size increase was observed (38 nm). This is likely due to the order of pH-responsive blocks in **P3₂₀₀**. With the carboxylic acid block directly connected to the P(**NB-MEG**) core, switching to pH 2 during the first step results in the carboxylic acid block burying itself within the core of the particle and the charged amine block remaining within the corona, preventing fusion from occurring due to electrostatic repulsion. Alternatively, when the switch to pH 2 is made following basification to pH 12, the amine-functionalised block is already buried within the core of a fused particle. This results in a screening effect of the positively charged amine groups and permits the further release of membrane tension by particle fusion. As a result, **P3₂₀₀** displays a significant pathway dependence when dual pH-triggered across two steps.

2.4 Conclusions

In this work, a carboxylic acid-functionalised norbornene monomer was successfully synthesised and incorporated into self-assembled nano-objects *via* ROMPISA. Variation in buffer pH utilised during polymerisation was shown to dictate resulting morphology, with spherical polymersomes formed at pH 4.5 and above, whilst fused tubular structures were the result of using acidic buffers at $\text{pH} \leq 3$. This difference in resulting morphology is attributed to the protonation of the carboxylic acid group at low pH, reducing electrostatic repulsion between colliding particles, thus reducing the energy barrier to fusion. In addition, the degree of polymersome fusion was influenced by either increasing the DP of the core block or reducing the buffer pH, both resulting in an interwoven network of fused tubes. Further to this, when a core DP of 300 was targeted using a pH 2 buffer, the resulting nano-object solution formed a free-standing gel.

Following this, carboxylic acid-functionalised polymersomes were realised in an unbuffered solution, where the addition of chloride ions facilitated polymerisation. For all core DPs, spherical nano-objects were formed, with average diameters between 18-24 nm. At neutral pH, it was anticipated that the carboxylate group would be deprotonated, resulting in negatively charged polymersomes that were prevented from fusing due to their electrostatic repulsion, despite possessing significant membrane tension. As such, the addition of HCl to reduce the pH resulted in the 1D triggered fusion of polymersomes into tubular structures, with the length of tube formed increasing with higher concentrations of acid solution.

Kinetic monitoring provided mechanistic insights into the fusion process, highlighting an inverse rate-dependence for carboxylic acid-containing polymersomes when compared to the

amine-functionalised analogues. This was further supported by the final morphology of linear tubes with minimal branching events, indicating a preference for uniaxial growth during polymersome fusion.

Through incorporation of both amine and carboxylic acid functionalities, dual pH-responsive polymersomes were successfully prepared. Both polymer microstructure and block order were shown to have no impact on the self-assembly process, with spherical nano-objects of between 15-20 nm characterised by dry-state TEM. Only a small degree of fusion was observed for all particles when switched to pH 12. However, block order had a greater impact when the particles were acidified, with the P(**NB-COOH**) block successfully burying itself into the core of the particle, irrespective of the presence of a hydrophilic amine block separating the two. From this, we inferred the hydrophobicity of the **NB-COOH** monomer when protonated is greater than the hydrophilicity of the amine-functionalised monomer at pH 2, allowing it to overcome the energy barrier to fusion.

Finally, the dual pH-responsive nature of the polymersomes was realised by initially switching the particle solutions to pH 12 before acidifying the solution to pH 2, and vice versa. Again, block order was determined as a key factor in final particle morphology. Polymer block orders that allowed for sequential insertion of the triggered blocks into the core of the particle resulted in the formation of linear tubes, whereas other systems formed morphologies of branched or aggregated structures.

These results broaden the scope of developing synthetic, out-of-equilibrium systems that are responsive to external stimuli and result in the combination of polymersomes. Inclusion of

two pH-responsive groups increases the selectivity of fusion within these systems and promotes development in system complexity that will advance this area further.

2.5 Experimental

2.5.1 Materials

Unless otherwise stated reagents were obtained from commercial sources and used without purification. THF (HPLC grade) was purchased from VWR Chemicals and was purified *via* passage through a column of neutral alumina prior to use. Formvar-carbon coated (300 mesh) copper grids were purchased from EM Resolutions.

G3 (modified Grubbs 3rd generation catalyst, $((\text{H}_2\text{IMes})(\text{pyr})_2(\text{Cl})_2\text{Ru}=\text{CHPh})$), **NB-PEG**, **NB-Amine**, **NB-MEG**, and exo-5-norbornene imide were prepared as previously described.^{15, 20, 32}

2.5.2 Characterisation Techniques

2.5.2.1 NMR Spectroscopy

^1H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker DPX-400 spectrometer in CDCl_3 or $(\text{CD}_3)_2\text{SO}$. Chemical shifts of protons are reported as δ in parts per million (ppm) and are relative to solvent residual peaks (CDCl_3 δ = 7.26 ppm, $(\text{CD}_3)_2\text{SO}$ δ = 2.50 ppm). All ^1H resonances are reported to the nearest 0.01 ppm. The multiplicity of ^1H signals are indicated as: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; or combinations of thereof. Coupling constants (J) are quoted in Hz and reported to the nearest 0.1 Hz. Where appropriate, averages of the signals from peaks displaying multiplicity were used to calculate the value of the coupling constant. ^{13}C NMR spectra were recorded on the same spectrometer with the central resonance of the solvent peak as the internal reference (CDCl_3 δ = 77.16 ppm). All ^{13}C resonances are reported to the nearest 0.01 ppm.

2.5.2.2 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) analysis was performed on a system composed of an Agilent 1260 Infinity II LC system equipped with an Agilent guard column (PLGel 5 μ M, 50 $\times$ 7.5 mm) and two Agilent Mixed-C columns (PLGel 5 μ M, 300 $\times$ 7.5 mm). The mobile phase used was THF (HPLC grade) containing 2% v/v NEt_3 at 40 $^\circ\text{C}$ with a flow rate of 1.0 mL min^{-1} (poly(methyl methacrylate) (PMMA) standards used for calibration). Number average molecular weight (M_n), weight average molecular weight (M_w) and dispersity ($\mathcal{D} = M_w/M_n$) were determined using Agilent SEC/SEC software.

2.5.2.3 Dynamic Light Scattering and Zeta Potential Analysis

Hydrodynamic diameters (D_h), size distributions (PD) and zeta potential (ζ) of nano-objects were determined by dynamic light scattering (DLS) and microelectrophoresis using a Malvern Zetasizer Nano ZS with a 4 mW He-Ne 633 nm laser module operating at 25 $^\circ\text{C}$. Samples were diluted to give a final polymer concentration of 0.01 wt%. Measurements were carried out at an angle of 173 $^\circ$ (back scattering), and results were analysed using Malvern DTS v7.03 software. All determinations were repeated five times with at least 10 measurements recorded for each run (50 measurements for zeta potential). D_h values were calculated using the Stokes-Einstein equation where particles are assumed to be spherical, while for anisotropic particles DLS was used to indicate a change in average particle size and obtain dispersity information. Zeta potential values were calculated from the corresponding electrophoretic mobilities (μ_e) by using the Henry's correction of the Smoluchowski equation.

2.5.2.4 Transmission Electron Microscopy

Dry-state, stained transmission electron microscopy (TEM) imaging was performed on a JEOL JEM-1400 microscope operating at an acceleration voltage of 80 kV. All dry-state samples were diluted to an appropriate analysis concentration and then deposited onto formvar-coated grids. After roughly 1 min, excess sample was blotted from the grid and the grid was then stained with an aqueous 1 wt% uranyl acetate (UA) solution for 1 min prior to blotting, drying and microscopic analysis. Images were analysed using ImageJ software in which a minimum of 100 particles across a minimum of 3 images were measured.

2.5.2.5 High-Resolution Mass Spectrometry

High-resolution mass spectrometry (HRMS) spectra were recorded by the MS Analytical Facility Service at the University of Birmingham on a Waters Xevo G2-XS QToF Quadrupole Time-of-Flight mass spectrometer.

2.5.3 Monomer Synthesis

2.5.3.1 Synthesis of *exo*-Norbornene imide carboxylic acid (NB-COOH).

exo-Norbornene anhydride (1.5 g, 9.14 mmol) and glycine (0.57 g, 7.61 mmol) were dissolved in 50 mL glacial acetic acid and the mixture was heated to 80 °C for 6 h. The reaction was quenched with water (50 mL). The crude product was then extracted with CH₂Cl₂ (50 mL × 3) and washed with saturated NaHCO₃ solution and water. The organic phases were combined, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (0 to 20% ethyl acetate in hexane) to yield **NB-COOH** (1.36 g, 6.15 mmol, 81%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 6.30 (t, *J* = 1.9 Hz, 2H), 4.28 (s, 2H), 3.32 (p, *J* = 1.7 Hz, 2H), 2.77 (d, *J* = 1.3 Hz, 2H), 1.61 (dt, *J* = 10.0, 1.5 Hz, 1H),

1.51 (dt, $J = 10.0, 1.6$ Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 177.31, 171.83, 138.14, 48.18, 45.56, 43.00, 39.23. MS: m/z $[\text{M}+\text{H}]^+$ calc. 222.08 g mol^{-1} , exp. 222.08 g mol^{-1} .

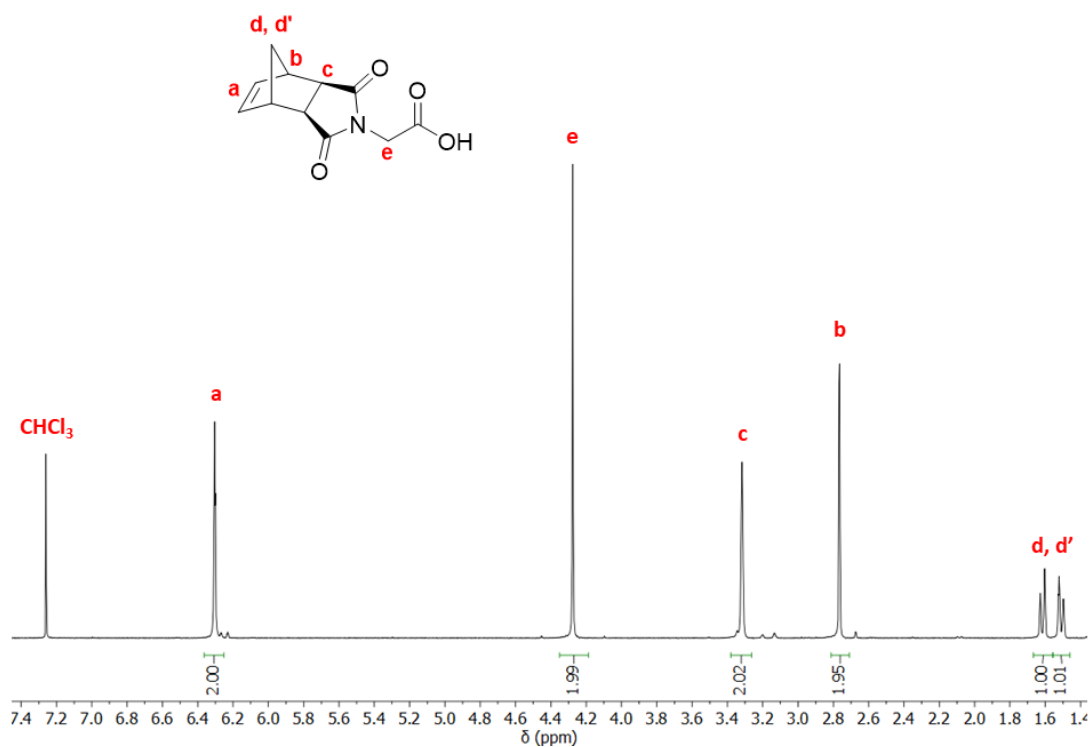


Figure S2.1. ^1H NMR spectrum (400 MHz, CDCl_3 , 298 K) of NB-COOH.

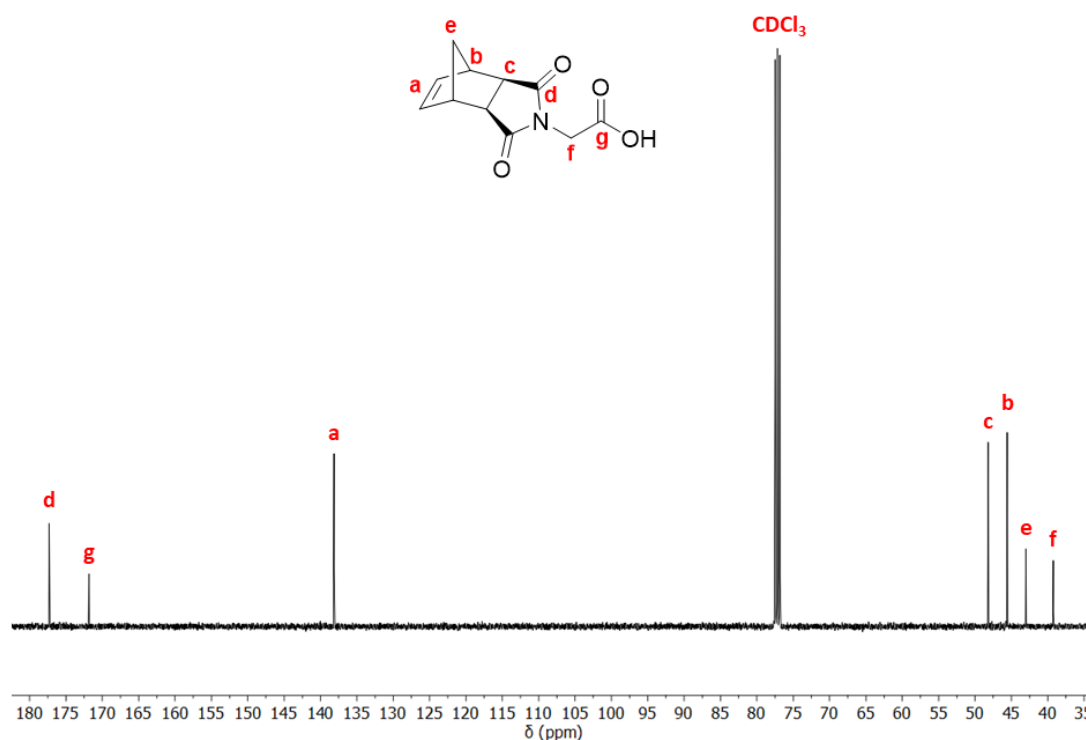


Figure S2.2. ^{13}C NMR spectrum (400 MHz, CDCl_3 , 298 K) of **NB-COOH**.

2.5.4 Synthesis and characterisation of carboxylic acid-containing triblock nano-objects *via* ROMPISA

2.5.4.1 $\text{P}(\text{NB-PEG})_{11}\text{-}b\text{-P}(\text{NB-COOH})_5\text{-}b\text{-P}(\text{NB-MEG})_x$ at pH 2, $\text{P}_{\text{pH}21x}$

A solution of **NB-PEG** (31.2 mg, 59 μmol , 13 eq.) in 530 μL of filtered THF was rapidly added to a solution of **G3** (3.9 mg, 5.2 μmol , 1.2 eq.) in 120 μL of THF contained within a glass vial equipped with a stirrer bar. The resulting solution was stirred rapidly for five minutes. After this time a 100 μL aliquot was removed for SEC analysis, leaving 1.0 eq. of $\text{P}(\text{NB-PEG})_{11}$ in 550 μL of THF. To this solution was added **NB-COOH** (5.0 mg, 23 μmol , 5.0 eq.) in 450 μL THF. The resulting solution was stirred for a further five minutes to give $\text{P}(\text{NB-PEG})_{11}\text{-}b\text{-P}(\text{NB-COOH})_5$ macroinitiator (final concentration = 4.4 $\mu\text{mol}/\text{mL}$).

Three aliquots (100 μL , 0.44 μmol , for **P1₁₀₀**, 50 μL , 0.22 μmol , for **P1₂₀₀** and 33 μL , 0.15 μmol , for **P1₃₀₀**) of the resulting solution of **P(NB-PEG)₁₁-b-P(NB-COOH)₅** in THF were dispensed into 2 mL glass vials containing a stirrer bar. Filtered THF was added to give 100 μL in total in each vial. A solution of **NB-MEG** (10 mg, 45 μmol , 100 eq. for **P1₁₀₀**, 200 eq. for **P1₂₀₀** and 300 eq. for **P1₃₀₀**) in 900 μL of acidic phosphate buffer (pH = 2, PB2, final solids concentration = 1 wt%) was added rapidly to each vial. The resulting solution was thoroughly mixed by drawing up the entire volume into the pipette tip and ejecting the liquid back into the vial three times. The ROMPISA polymerisations were stirred at 420 rpm for 30 minutes to give **P(NB-PEG)₁₁-b-P(NB-COOH)₅-b-P(NB-MEG)_{100/200/300}** (**P_{pH2}1₁₀₀**, **P_{pH2}1₂₀₀** and **P_{pH2}1₃₀₀**, respectively). These were analysed by ^1H NMR spectroscopy, SEC, DLS and TEM.



Figure S2.3. Inverted vial test of **P_{pH2}1_x**, from left to right x = 100, 200 and 300.

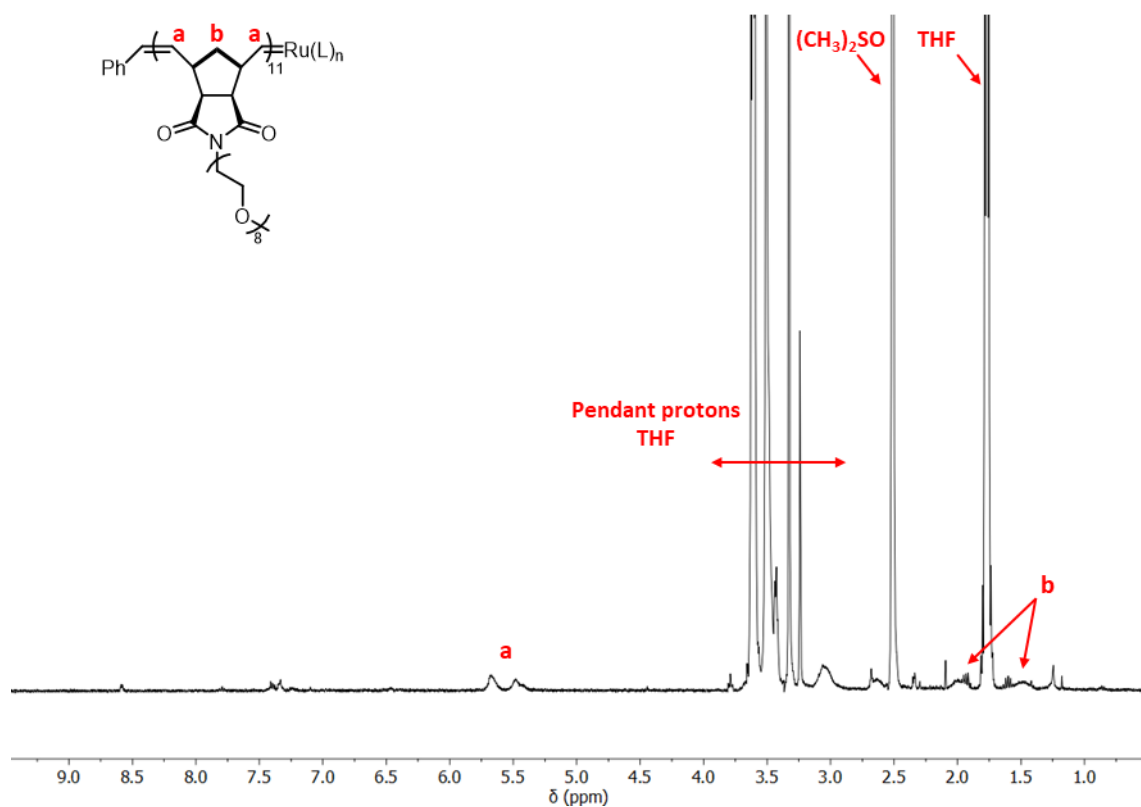


Figure S2.4. ^1H NMR spectrum (400 MHz, $(\text{CD}_3)_2\text{SO}$, 298 K) of $P(\text{NB-PEG})_{11} + \text{THF}$.

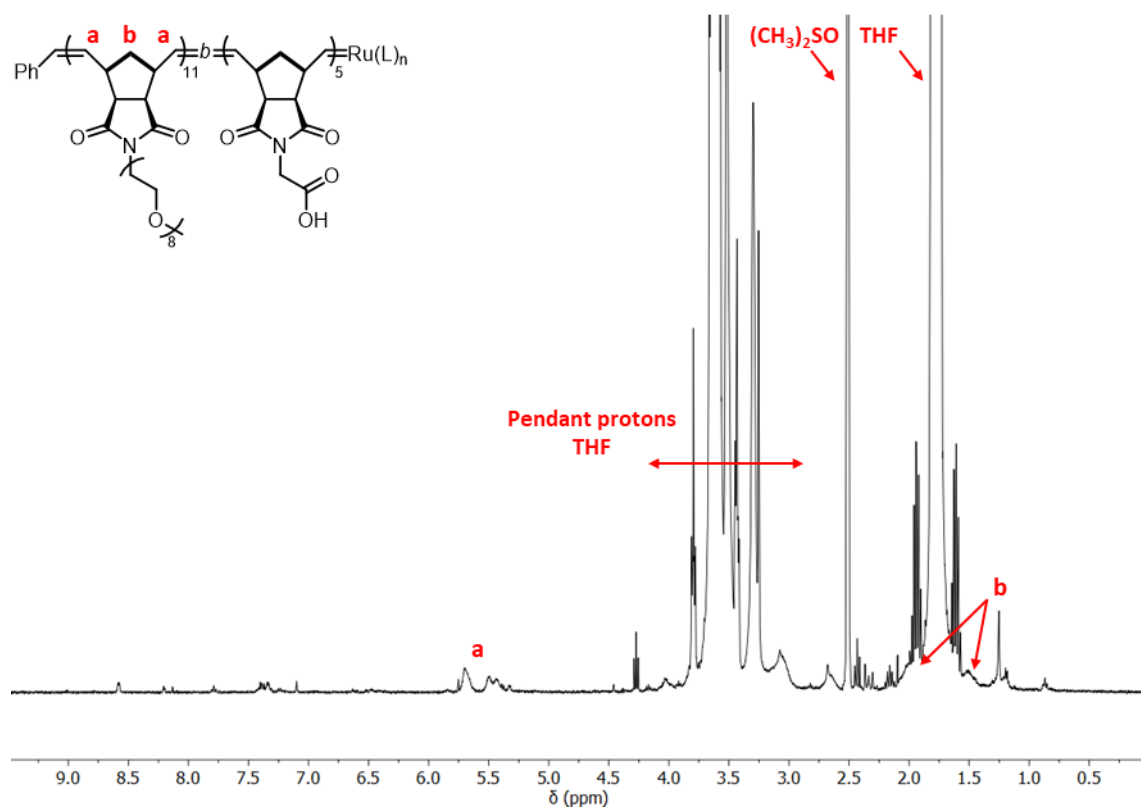


Figure S2.5. ^1H NMR spectrum (400 MHz, $(\text{CD}_3)_2\text{SO}$, 298 K) of $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-COOH})_5 + \text{THF}$.

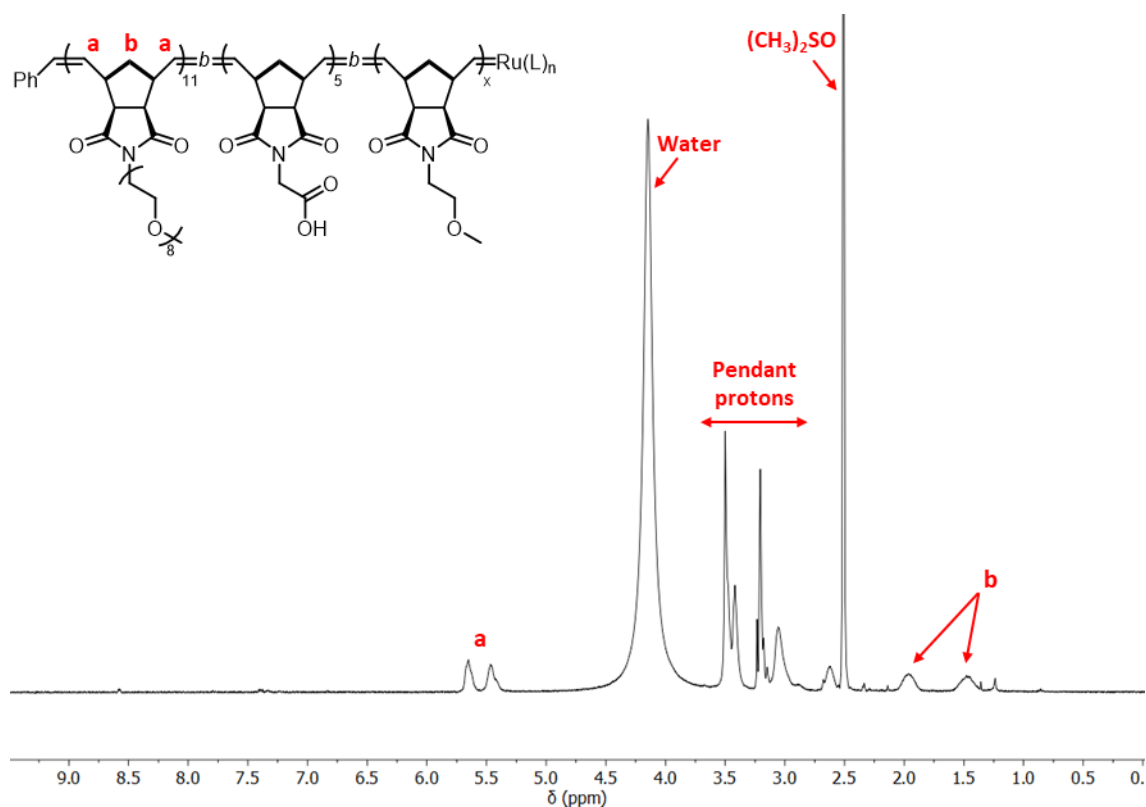


Figure S2.6. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P_{pH2}1_{100}$ + THF/PB2.

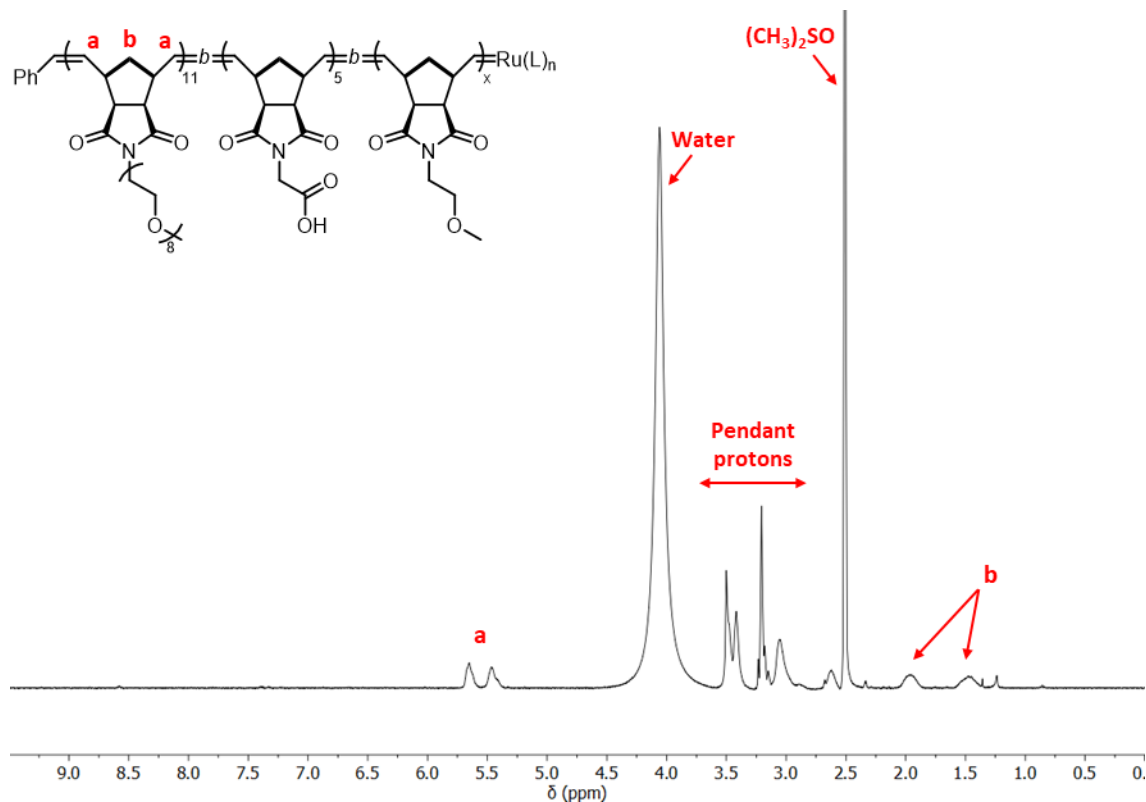


Figure S2.7. ^1H NMR spectrum (400 MHz, $(CD_3)_2SO$, 298 K) of $P_{pH2}1_{200}$ + THF/PB2.

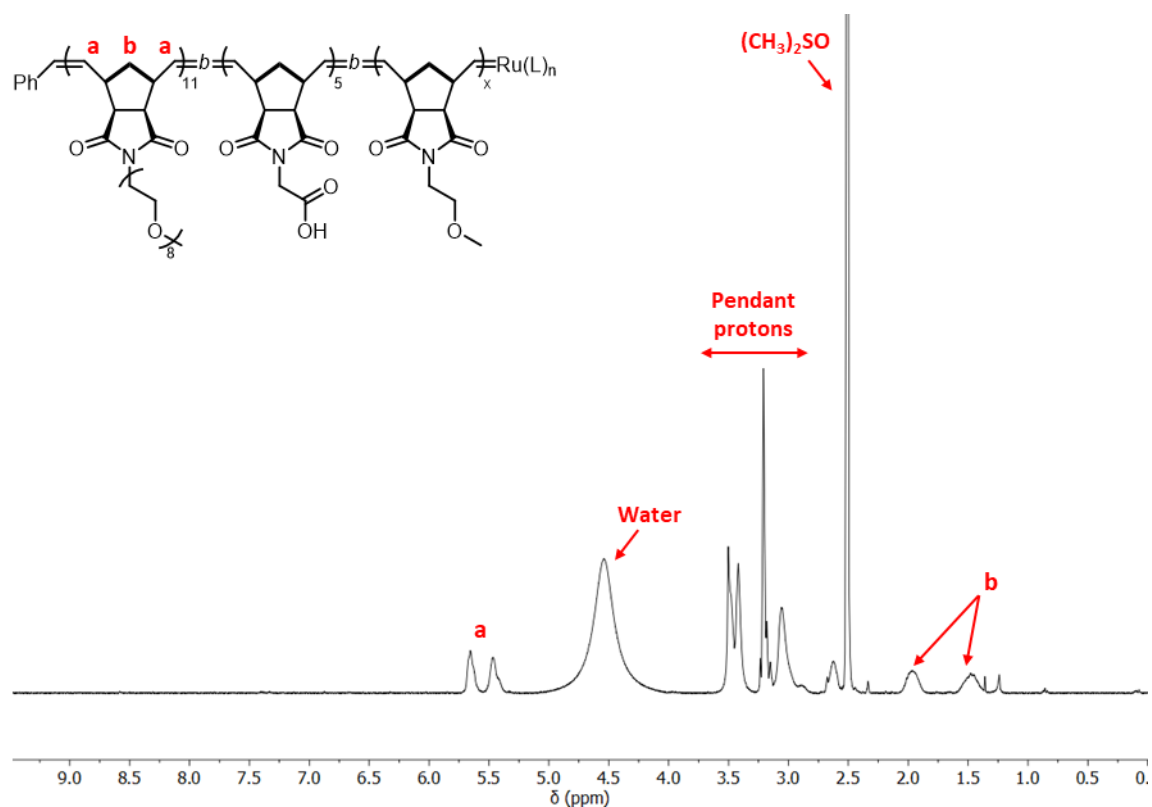


Figure S2.8. ^1H NMR spectrum (400 MHz, $(\text{CD}_3)_2\text{SO}$, 298 K) of $\text{P}_{\text{pH}2}\mathbf{1}_{300}$ + THF/PB2.

Table S2.1. SEC data of intermediate polymers for $\text{P}_{\text{pH}2}\mathbf{1}_x$.

Polymer	$M_{n,\text{theo}}$ / kDa	$M_{n,\text{SEC}}$ / kDa	$\bar{D}_{\text{SEC}}$
$\text{P}(\text{NB-PEG})_{11}$	6.5	7.4	1.13
$\text{P}(\text{NB-PEG})_{11}\text{-}b\text{-P}(\text{NB-COOH})_5$	7.6	8.0	1.14

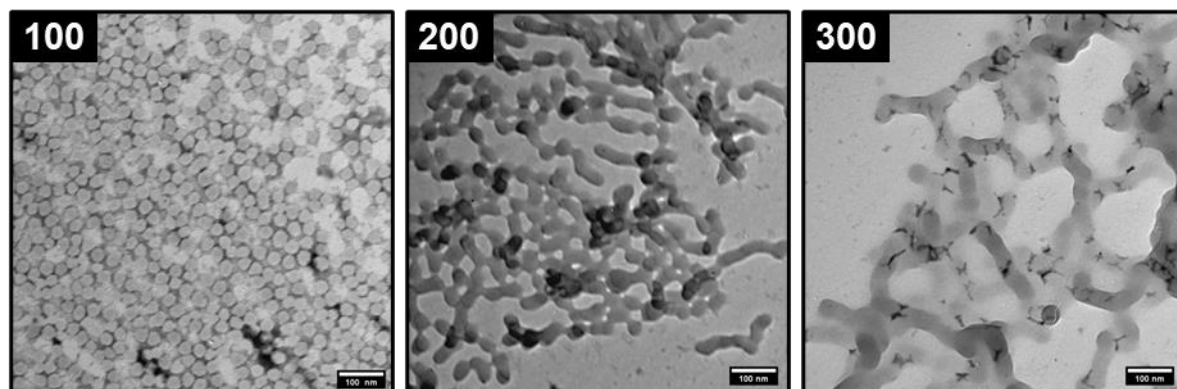


Figure S2.9. Supplementary dry-state TEM images of $P_{pH2}1_x$ particles: $x = 100$, 200 , and 300 . Scale bar = 100 nm.

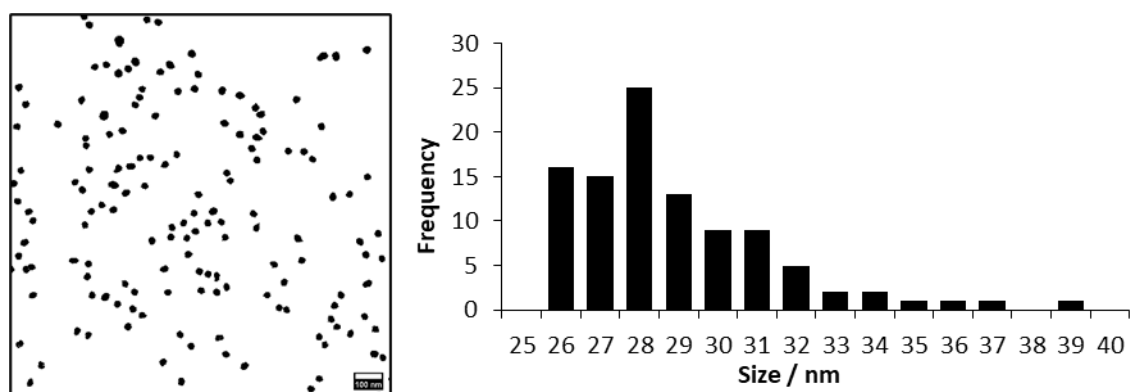


Figure S2.10. Dry-state TEM image analysis results for $P_{pH2}1_{100}$ particles, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.

2.5.4.2 $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-COOH})_5\text{-}b\text{-}P(\text{NB-MEG})_x$ in different pH phosphate buffers,

$P_{\text{pH}y}1_{200}$

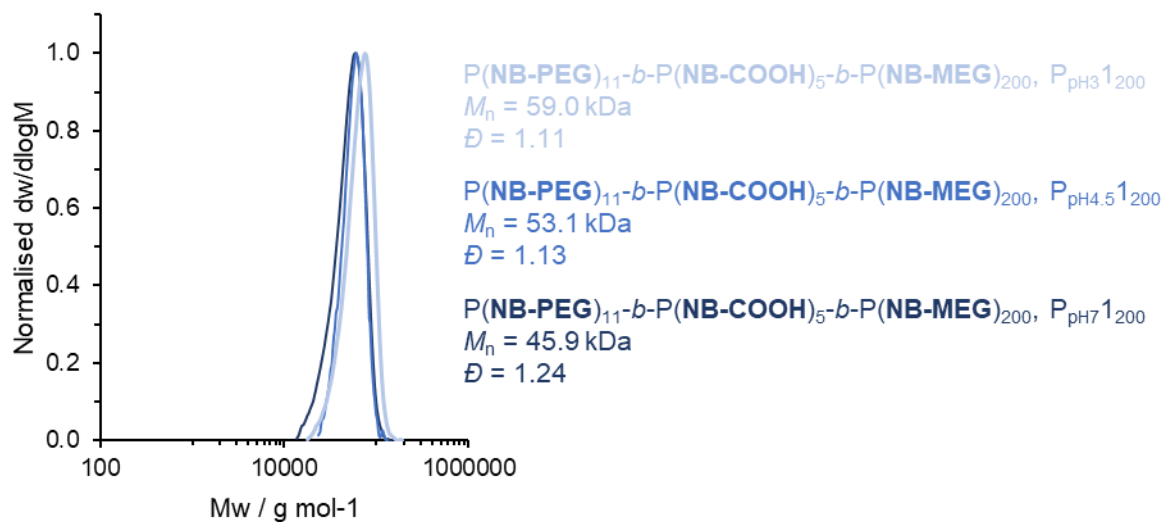


Figure S2.11. Normalised SEC traces (THF eluent, PMMA standards) of $P_{\text{pH}y}1_{200}$.

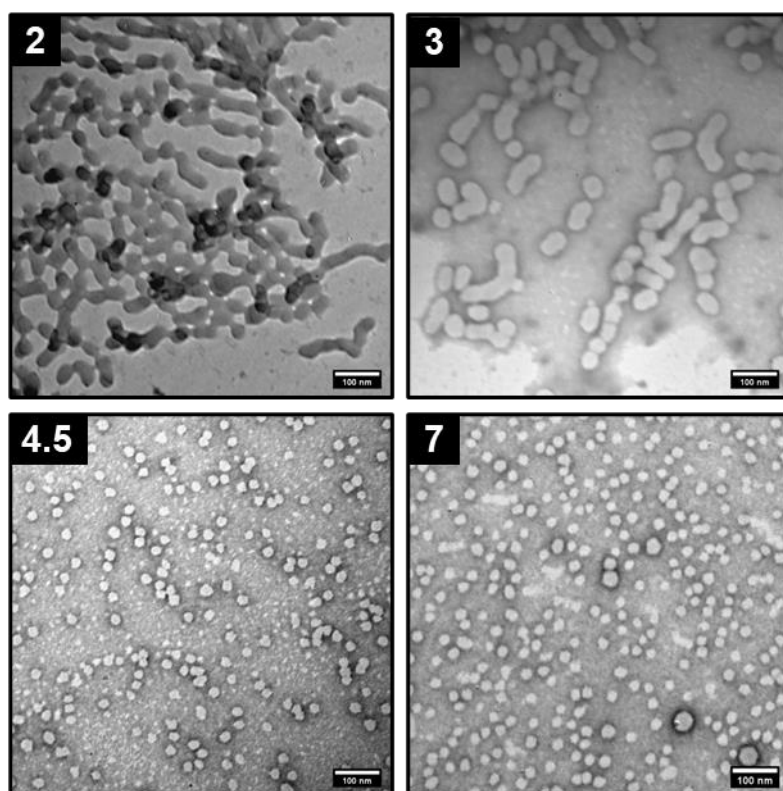


Figure S2.12. Supplementary dry-state TEM images of $P_{\text{pH}y}1_{200}$ particles synthesised at pH 2, 3, 4.5, and

7. Scale bar = 100 nm.

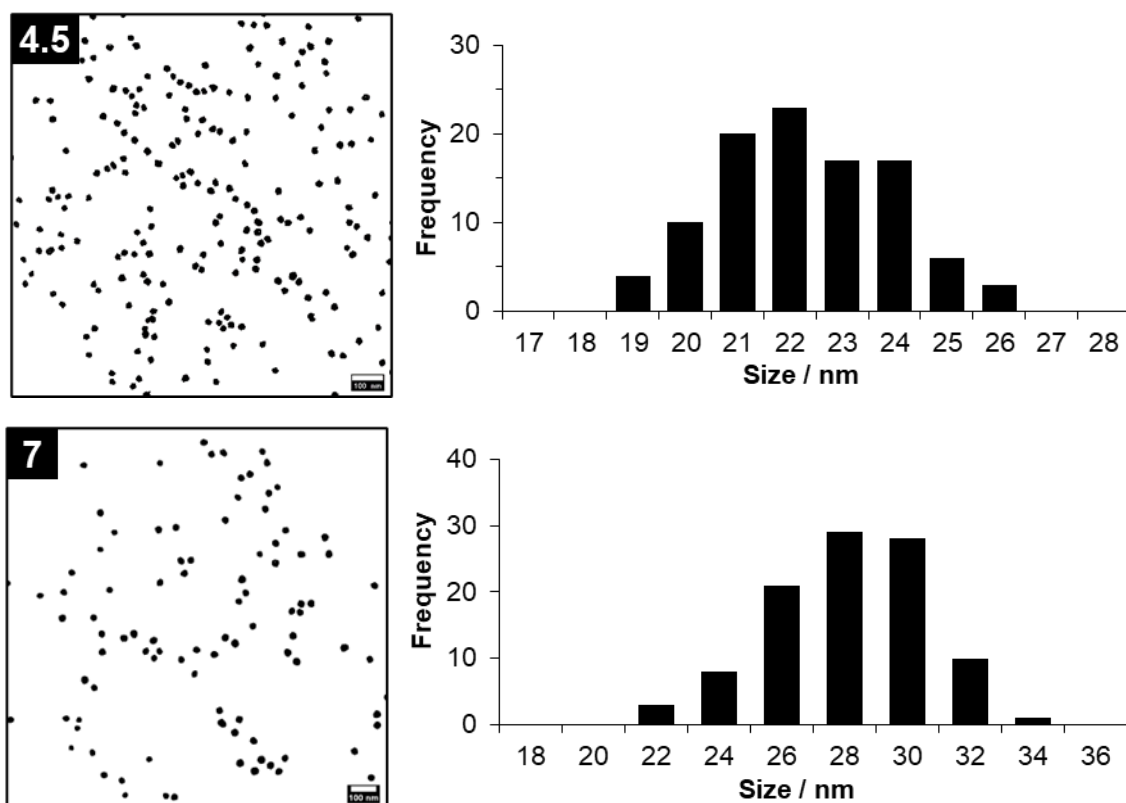


Figure S2.13 Dry-state TEM image analysis results for $P_{pHy1200}$ particles: pH 4.5 and 7, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.

2.5.4.3 $P(NB-PEG)_{11}-b-P(NB-COOH)_5-b-P(NB-MEG)_x$ in salt solution, $P1_x$

$P1_x$ nano-objects were synthesised analogous to $P_{pH2}1_x$, as described above.

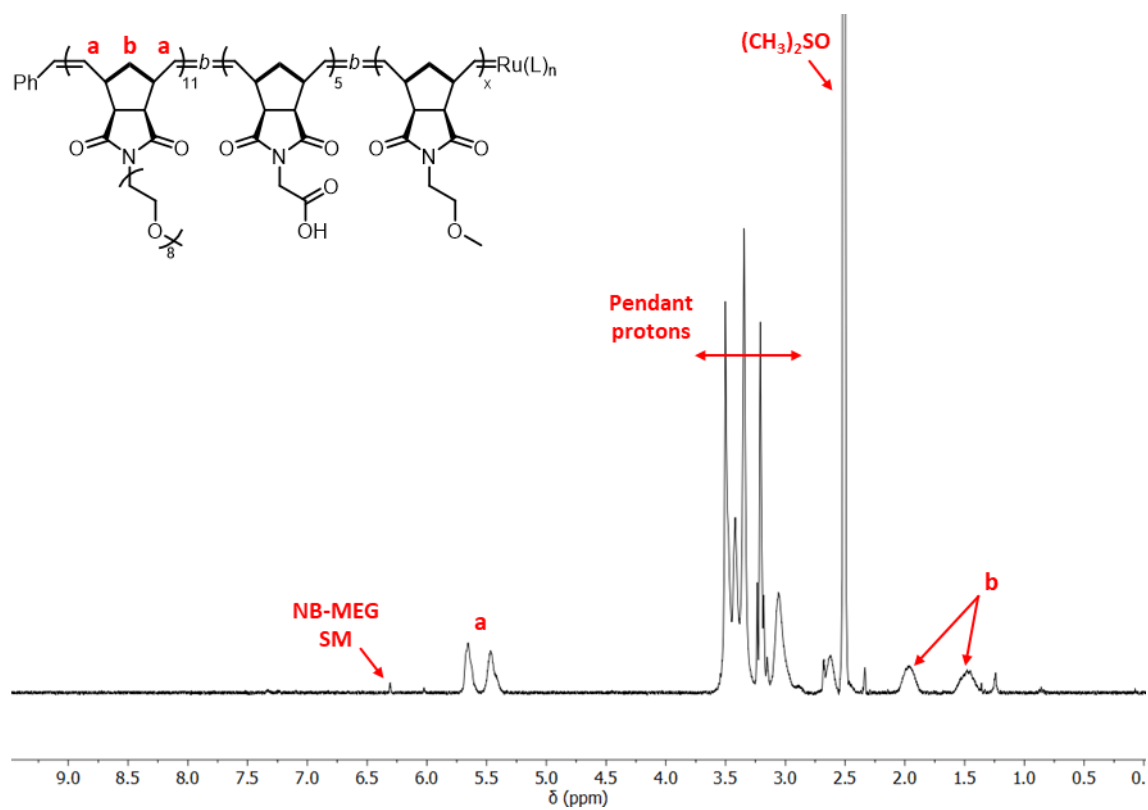


Figure S2.14. ^1H NMR spectrum (400 MHz, $(\text{CD}_3)_2\text{SO}$, 298 K) of $\mathbf{P1}_{100}$ + THF/AqNaCl.

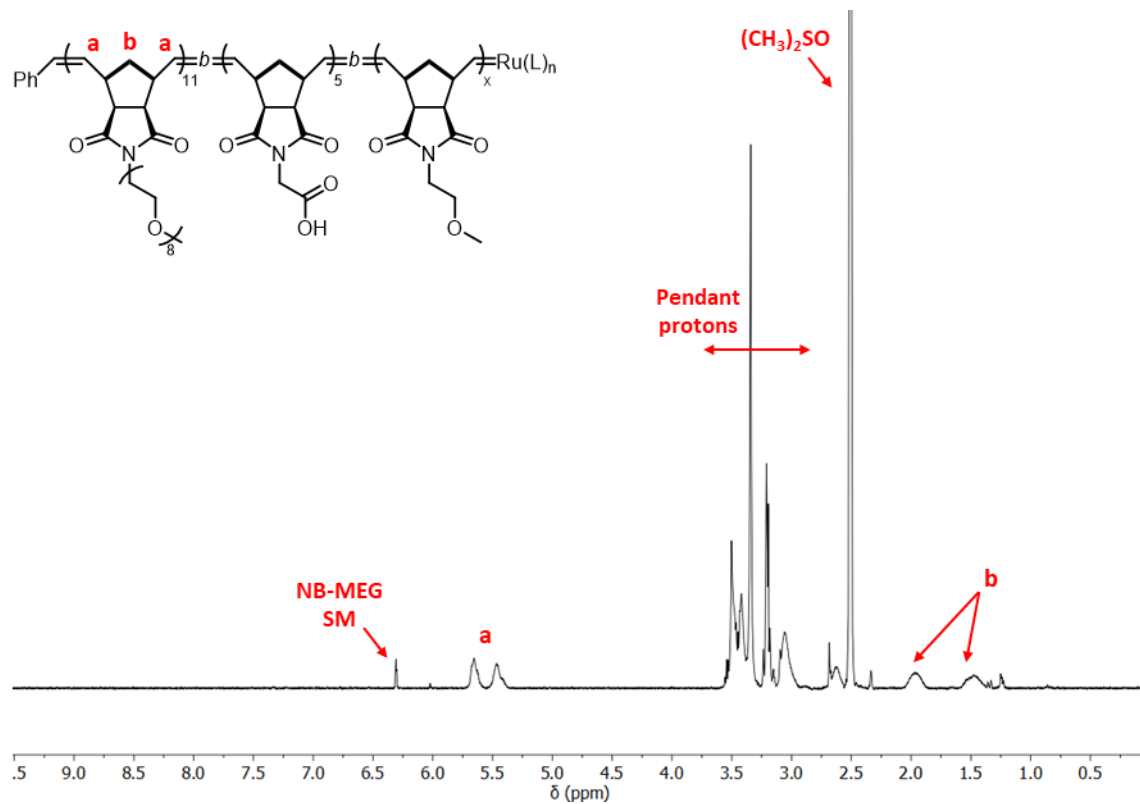


Figure S2.15. ^1H NMR spectrum (400 MHz, $(\text{CD}_3)_2\text{SO}$, 298 K) of $\mathbf{P1}_{200}$ + THF/AqNaCl.

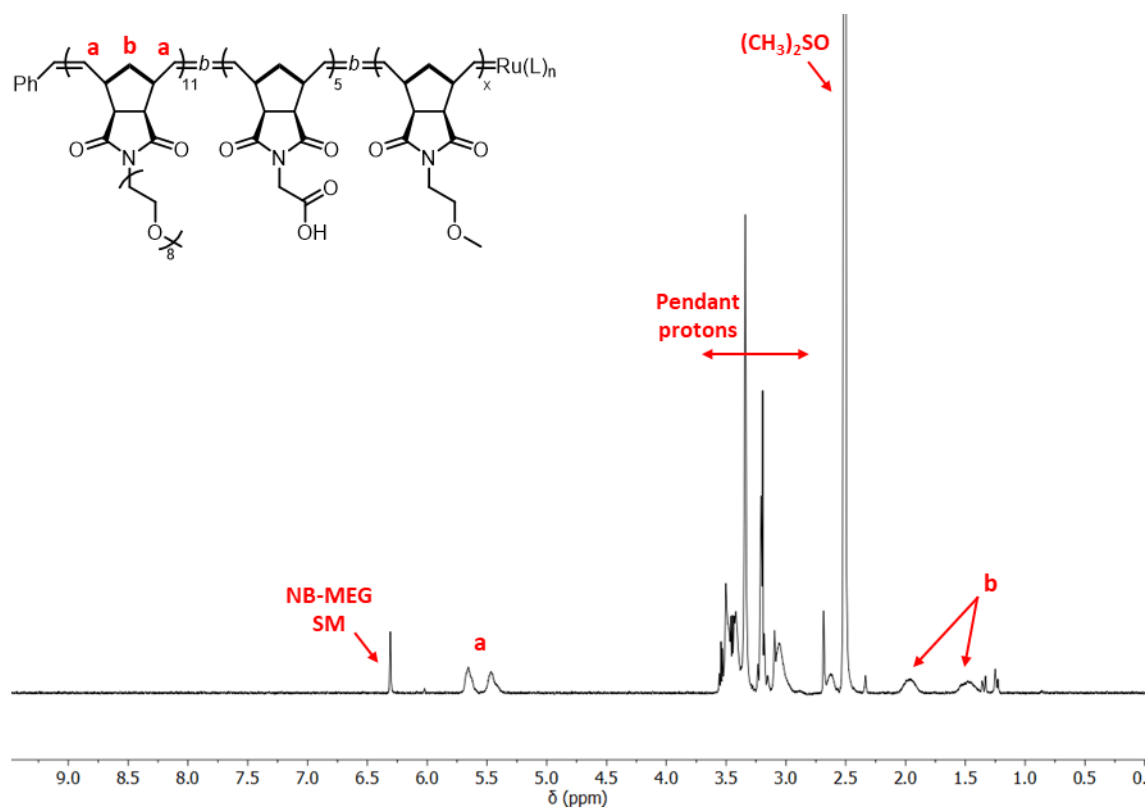


Figure S2.16. ^1H NMR spectrum (400 MHz, $(\text{CD}_3)_2\text{SO}$, 298 K) of $\text{P1}_{300} + \text{THF}/\text{Aq}_{\text{NaCl}}$.

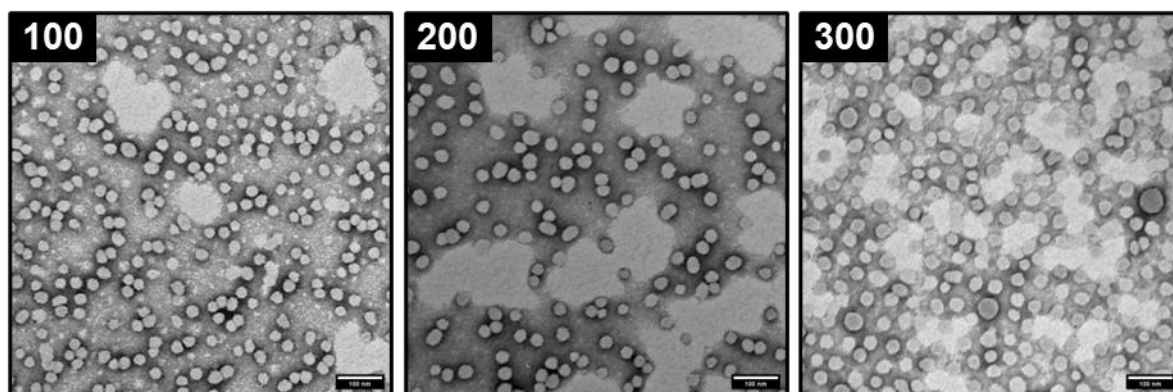


Figure S2.17. Supplementary dry-state TEM images of P1_x particles: $x = 100$, 200 , and 300 . Scale bar = 100 nm.

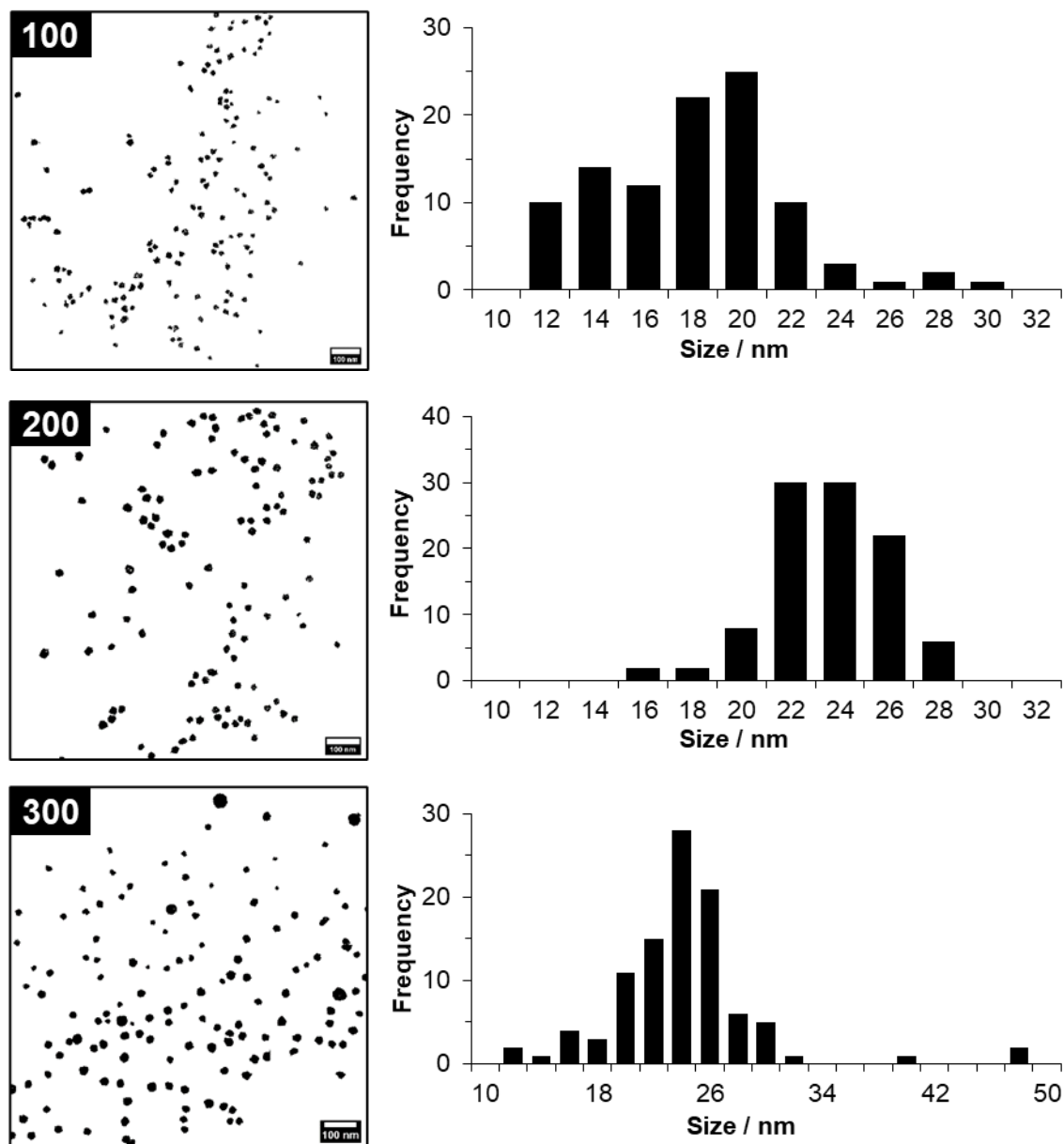


Figure S2.18. Dry-state TEM image analysis results for **P1_x** particles: $x = 100, 200$, and 300 , as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.

2.5.5 Triggered fusion of carboxylic acid-containing triblock nano-objects

2.5.5.1 Triggered fusion of **P1₂₀₀** using different concentrations of acid

To 100 μL solution of **P1₂₀₀** (as synthesised, 10 vol% THF in 100 mM NaCl) was rapidly added 300 μL of HCl solution (10 vol% THF). The resulting solution was thoroughly mixed by drawing

up the entire volume into the pipette tip and ejecting the liquid back into the vial three times.

The resulting solution was analysed by DLS and TEM.

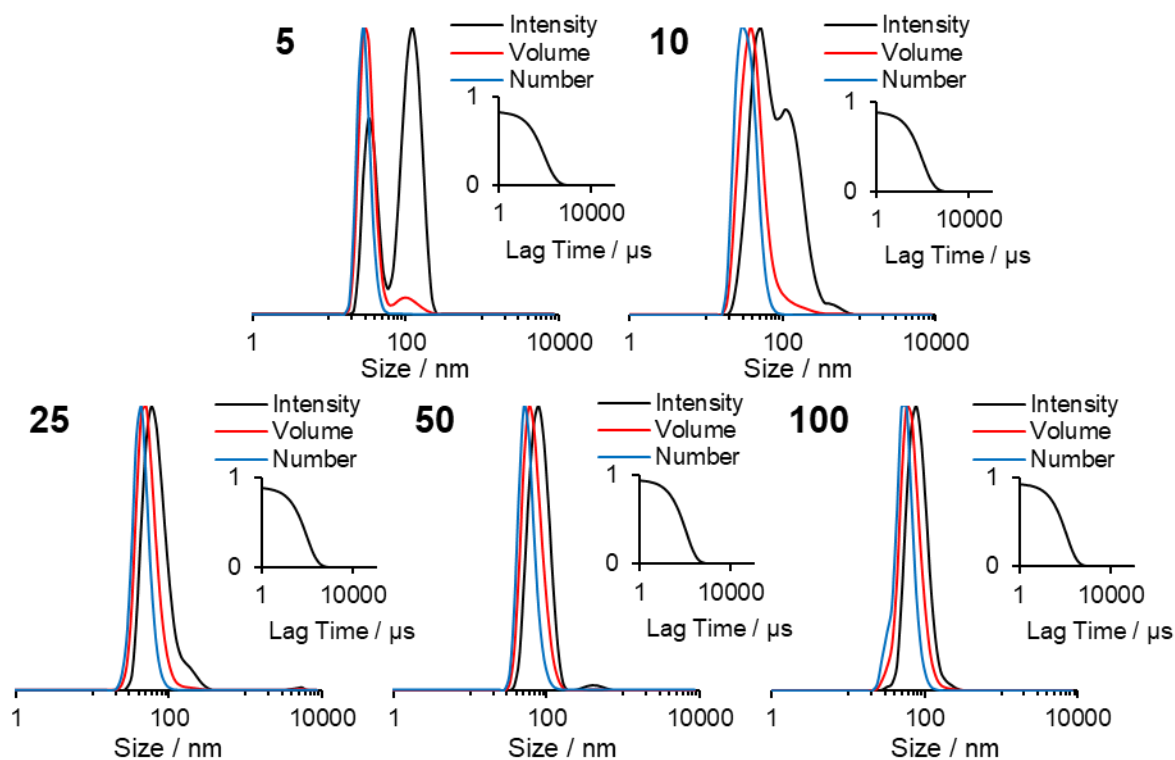


Figure S2.19. Normalised DLS analysis of **P1₂₀₀** particles acidified with different concentrations of HCl solution (+ 10% THF): 5, 10, 25, 50, and 100 mM.

2.5.5.2 Triggered fusion of **P1_x**

To 100 μ L solution of **P1_x** (as synthesised, 10 vol% THF in 100 mM NaCl) was rapidly added 300 μ L of HCl solution (50 mM, 10 vol% THF). The resulting solution was thoroughly mixed by drawing up the entire volume into the pipette tip and ejecting the liquid back into the vial three times. The resulting solution was analysed by DLS and TEM.

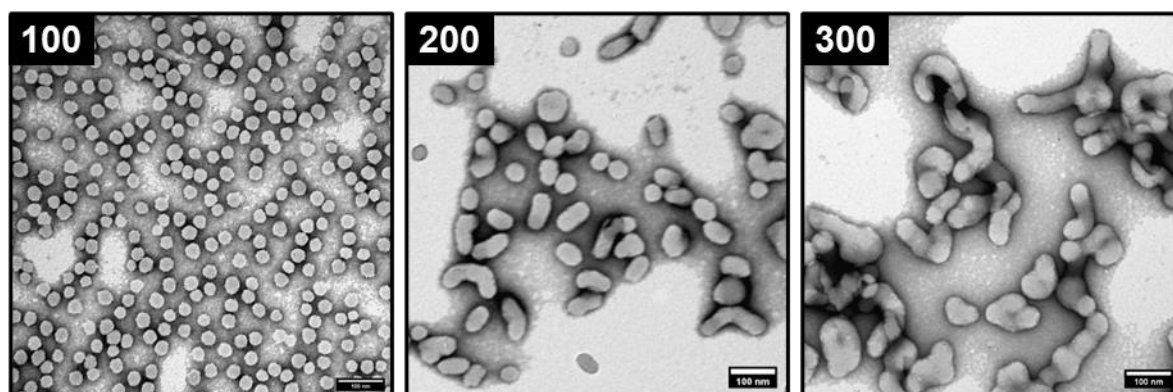


Figure S2.20. Supplementary dry-state TEM images of $P1_x$ particles synthesised at pH 7 and acidified to pH 2: $x = 100$, 200, and 300. Scale bar = 100 nm.

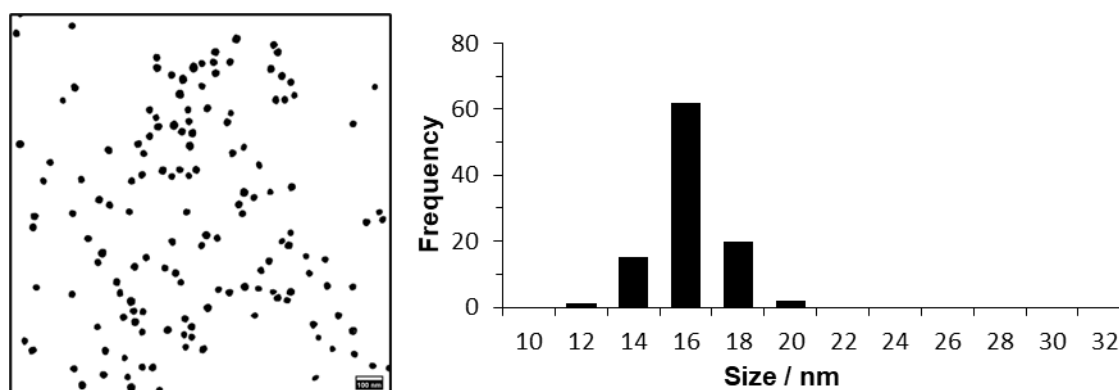


Figure S2.21. Dry-state TEM image analysis results for $P1_{100}$ particles acidified to pH 2, as determined by image processing using ImageJ analysis software. Scale bar = 100 nm.

2.5.5.3 Kinetics of triggered fusion of P1₂₀₀

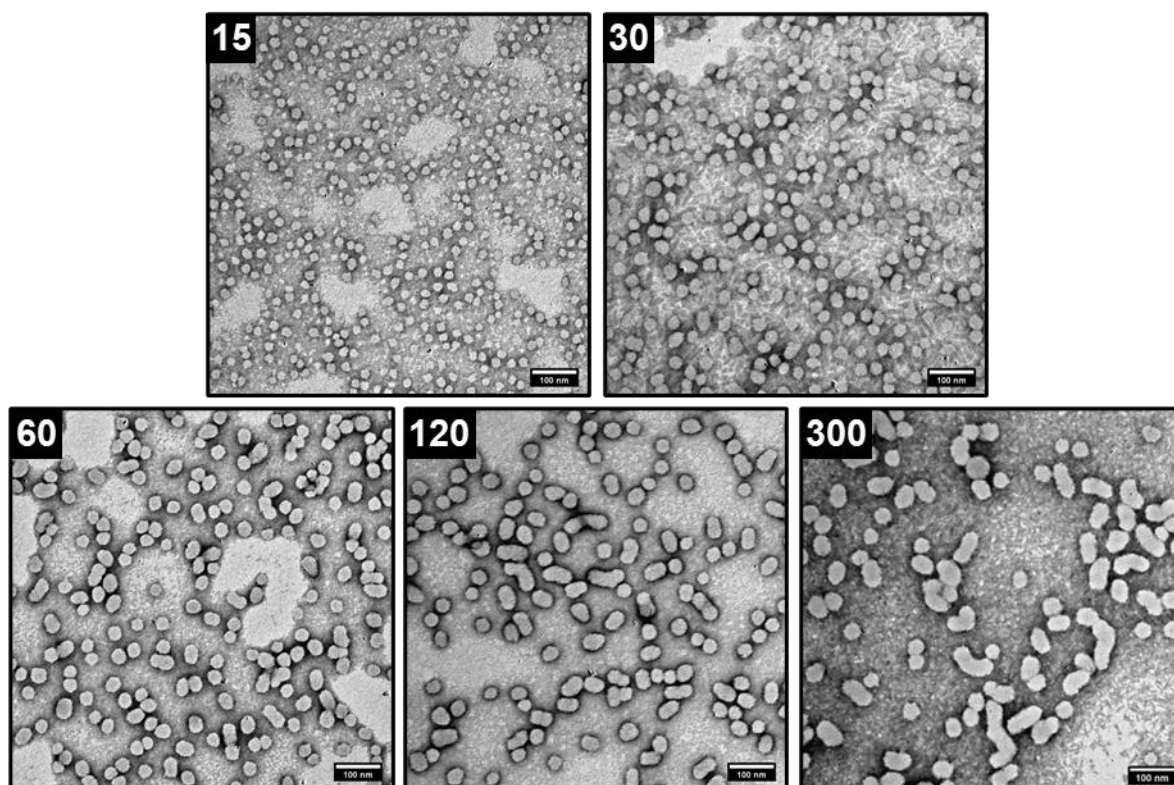


Figure S2.22. Supplementary dry-state TEM images of **P1₂₀₀** particles synthesised at pH 7 and acidified to pH 2, analysed at different time points: 15, 30, 60, 120, and 300 minutes. Scale bar = 100 nm.

The figure illustrates the synthesis of three polymers, P200, P300, and P400, through a series of reactions. The starting material is a polymer chain with a phenyl group (Ph) and a repeating unit (11) containing a ruthenium complex (Ru(L)_n).

Synthesis of P200:

- G3** reacts with **NB-PEG** in THF for 5 minutes to form a polymer with a PEG chain (8).
- This intermediate reacts with **NB-COOH** in THF for 5 minutes to form a polymer with a carboxylate group (5).
- The intermediate reacts with **NB-Amine** in THF for 5 minutes to form a polymer with a tertiary amine group (5).
- The intermediate reacts with **NB-MEG** in 100 mM AqNaCl / THF 9:1 for 30 minutes to form **P200**, which has a methoxy group (8).

Synthesis of P300:

- G3** reacts with **NB-PEG** in THF for 5 minutes to form a polymer with a PEG chain (8).
- This intermediate reacts with **NB-Amine** in THF for 5 minutes to form a polymer with a tertiary amine group (5).
- The intermediate reacts with **NB-COOH** in THF for 5 minutes to form a polymer with a carboxylate group (5).
- The intermediate reacts with **NB-MEG** in 100 mM AqNaCl / THF 9:1 for 30 minutes to form **P300**, which has a methoxy group (8).

Synthesis of P400:

- IB-PEG** reacts with **NB-Amine** in THF for 5 minutes to form a polymer with a tertiary amine group (5).
- This intermediate reacts with **NB-COOH** in THF for 5 minutes to form a polymer with a carboxylate group (5).
- The intermediate reacts with **NB-MEG** in 100 mM AqNaCl / THF 9:1 for 30 minutes to form **P400**, which has a methoxy group (8).

A solution of **NB-PEG** (31.2 mg, 59 μmol , 13 eq.) in 530 μL of filtered THF was rapidly added to a solution of **G3** (3.8 mg, 5.2 μmol , 1.2 eq.) in 120 μL of THF contained within a glass vial equipped with a stirrer bar. The resulting solution was stirred rapidly for five minutes to give **P(NB-PEG)₁₁**. After this time a 100 μL aliquot was removed for SEC analysis, leaving 1.0 eq. of **P(NB-PEG)₁₁** in 550 μL of THF. To this solution was added **NB-COOH** (2.6 mg, 11 μmol , 2.5 eq.) in 450 μL THF. The resulting solution was stirred for a further five minutes to give **P(NB-PEG)₁₁-b-P(NB-COOH)_{2.5}**. After this time a 100 μL aliquot was removed for SEC analysis, leaving 0.9

eq. of $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-COOH})_{2.5}$ in 900 μL of THF. To this solution was added **NB-Amine** (2.4 mg, 11 μmol , 2.3 eq.) in 450 μL THF. The resulting solution was stirred for a further five minutes to give $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-COOH})_{2.5}\text{-}b\text{-}P(\text{NB-Amine})_{2.5}$ macroinitiator (final concentration = 3.3 $\mu\text{mol/mL}$).

An aliquot (68 μL , 0.22 μmol) of the resulting solution of $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-COOH})_{2.5}\text{-}b\text{-}P(\text{NB-Amine})_{2.5}$ in THF was dispensed into a 2 mL glass vial containing a stirrer bar. Filtered THF and 100 mM NaCl solution was added to give 100 μL in total of each. A solution of **NB-MEG** (10 mg, 45 μmol) in 0.8 mL of 100 mM NaCl solution (pH = 7, final solids concentration = 1 wt%) was added rapidly. The resulting solution was thoroughly mixed by drawing up the entire volume into the pipette tip and ejecting the liquid back into the vial. The ROMPISA polymerisations were stirred at 300 rpm for 30 minutes to give $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-COOH})_{2.5}\text{-}b\text{-}P(\text{NB-Amine})_{2.5}\text{-}b\text{-}P(\text{NB-MEG})_{200}$ (**P2₂₀₀**). This was analysed by SEC, DLS and TEM.

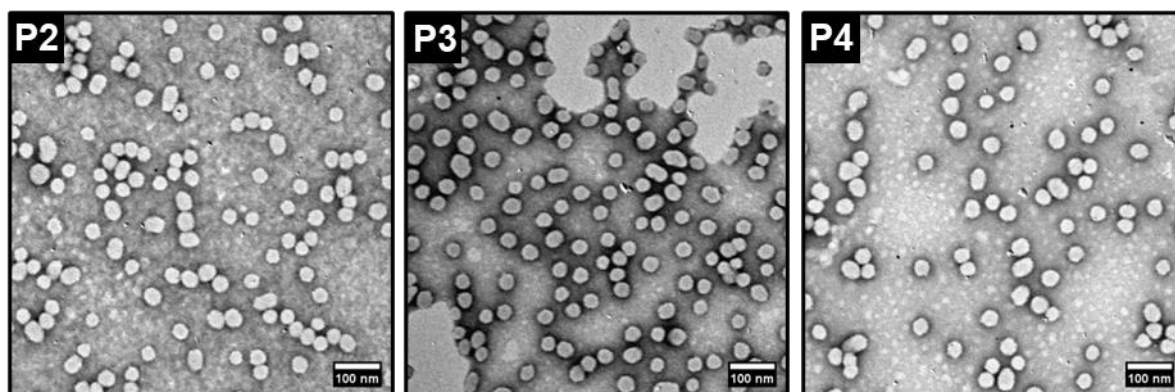
The order of addition for **NB-COOH** and **NB-Amine** were reversed to produce $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-Amine})_{2.5}\text{-}b\text{-}P(\text{NB-COOH})_{2.5}\text{-}b\text{-}P(\text{NB-MEG})_{200}$ (**P3₂₀₀**). Both **NB-COOH** and **NB-Amine** were added simultaneously to give $P(\text{NB-PEG})_{11}\text{-}b\text{-}(P(\text{NB-COOH})_{2.5}\text{-}r\text{-}P(\text{NB-Amine})_{2.5})\text{-}b\text{-}P(\text{NB-MEG})_{200}$ (**P4₂₀₀**).

Table S2.2. Polymer composition of zwitterionic quadrablock nano-objects synthesised *via* ROMPISA.

Polymer	Corona Block	pH-Responsive Block	Core Block
P2₂₀₀	$P(\text{NB-PEG})_{11}$	$P(\text{NB-COOH})_{2.5}\text{-}b\text{-}P(\text{NB-Amine})_{2.5}$	$P(\text{NB-MEG})_{200}$
P3₂₀₀	$P(\text{NB-PEG})_{11}$	$P(\text{NB-Amine})_{2.5}\text{-}b\text{-}P(\text{NB-COOH})_{2.5}$	$P(\text{NB-MEG})_{200}$
P4₂₀₀	$P(\text{NB-PEG})_{11}$	$P(\text{NB-Amine})_{2.5}\text{-}r\text{-}P(\text{NB-COOH})_{2.5}$	$P(\text{NB-MEG})_{200}$

Table S2.3. SEC data of intermediate polymers for **P2₂₀₀**, **P3₂₀₀** and **P4₂₀₀**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,SEC}$ / kDa	$\bar{D}_{SEC}$
P(NB-PEG) ₁₁	6.5	5.7	1.13
P(NB-PEG) ₁₁ - <i>b</i> -P(NB-COOH) _{2.5}	7.0	5.1	1.19
P(NB-PEG) ₁₁ - <i>b</i> -P(NB-Amine) _{2.5}	7.1	6.5	1.13
P(NB-PEG) ₁₁ - <i>b</i> -P(NB-COOH) _{2.5} - <i>b</i> -P(NB-Amine) _{2.5}	7.6	5.6	1.13
P(NB-PEG) ₁₁ - <i>b</i> -P(NB-Amine) _{2.5} - <i>b</i> -P(NB-COOH) _{2.5}	7.6	5.5	1.12
P(NB-PEG) ₁₁ - <i>b</i> -(P(NB-COOH) _{2.5} - <i>r</i> -P(NB-Amine) _{2.5})	7.6	5.3	1.13

**Figure S2.23.** Supplementary dry-state TEM images of **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀**. Scale bar = 100 nm.

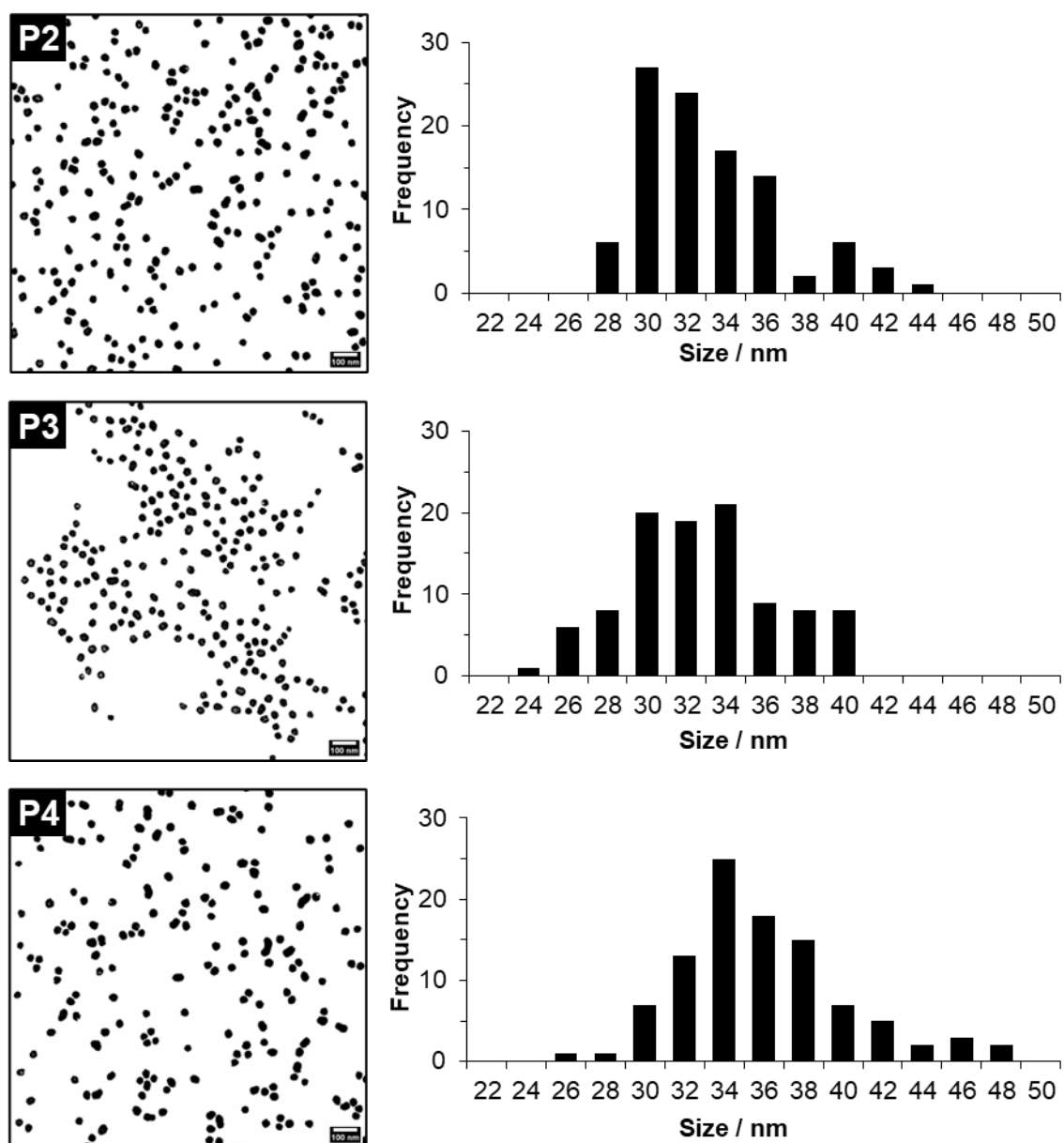


Figure S2.24. Dry-state TEM image analysis results for **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀**, as determined by image processing using ImageJ analysis software.

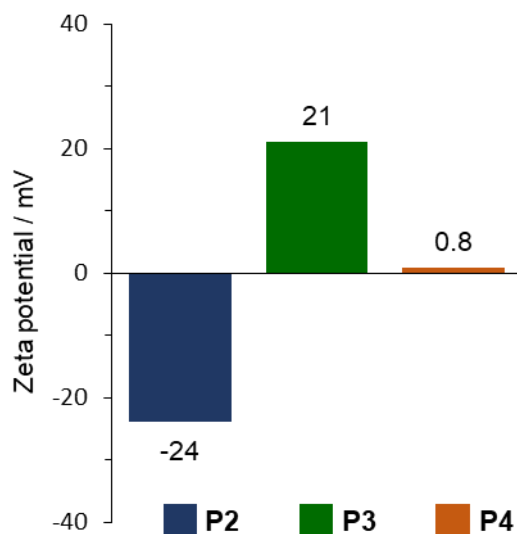


Figure S2.25. Zeta potential values for **P2₂₀₀** (blue), **P3₂₀₀** (green), and **P4₂₀₀** (orange) synthesised at pH 7, measured from microelectrophoretic analysis.

2.5.7 Dual pH triggering of zwitterionic nano-objects

To 100 μL solution of **P2₂₀₀** (as synthesised, 10 vol% THF in 100 mM NaCl) was rapidly added 100 μL of HCl solution (50 mM, 10 vol% THF). The resulting solution was thoroughly mixed by drawing up the entire volume into the pipette tip and ejecting the liquid back into the vial three times. A 20 μL aliquot was removed for analysis. Following this, to 200 μL of NaOH solution (100 mM, 10 vol% THF) was added to the acidified mixture. Again, the resulting solution was thoroughly mixed by drawing up the entire volume into the pipette tip and ejecting the liquid back into the vial three times. The resulting solution was analysed by DLS and TEM. This process was repeated for both **P3₂₀₀** and **P4₂₀₀**.

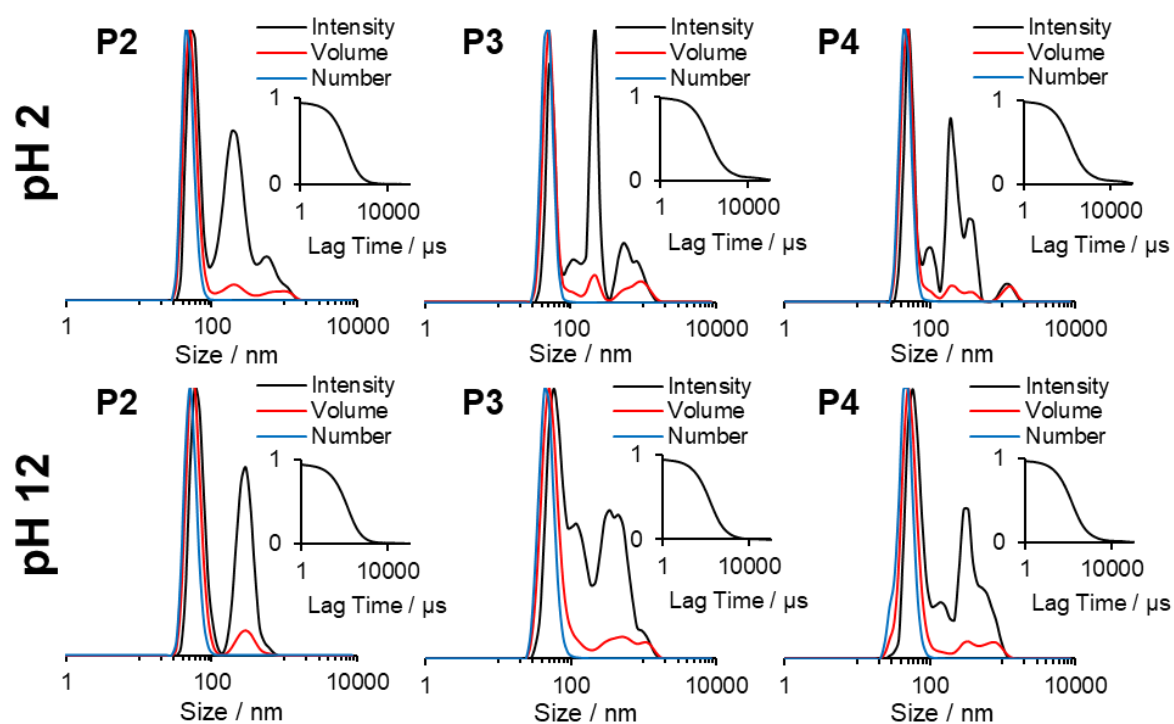


Figure S2.26. Normalised DLS analysis of **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀** particles acidified to pH 2 and then switched to pH 12.

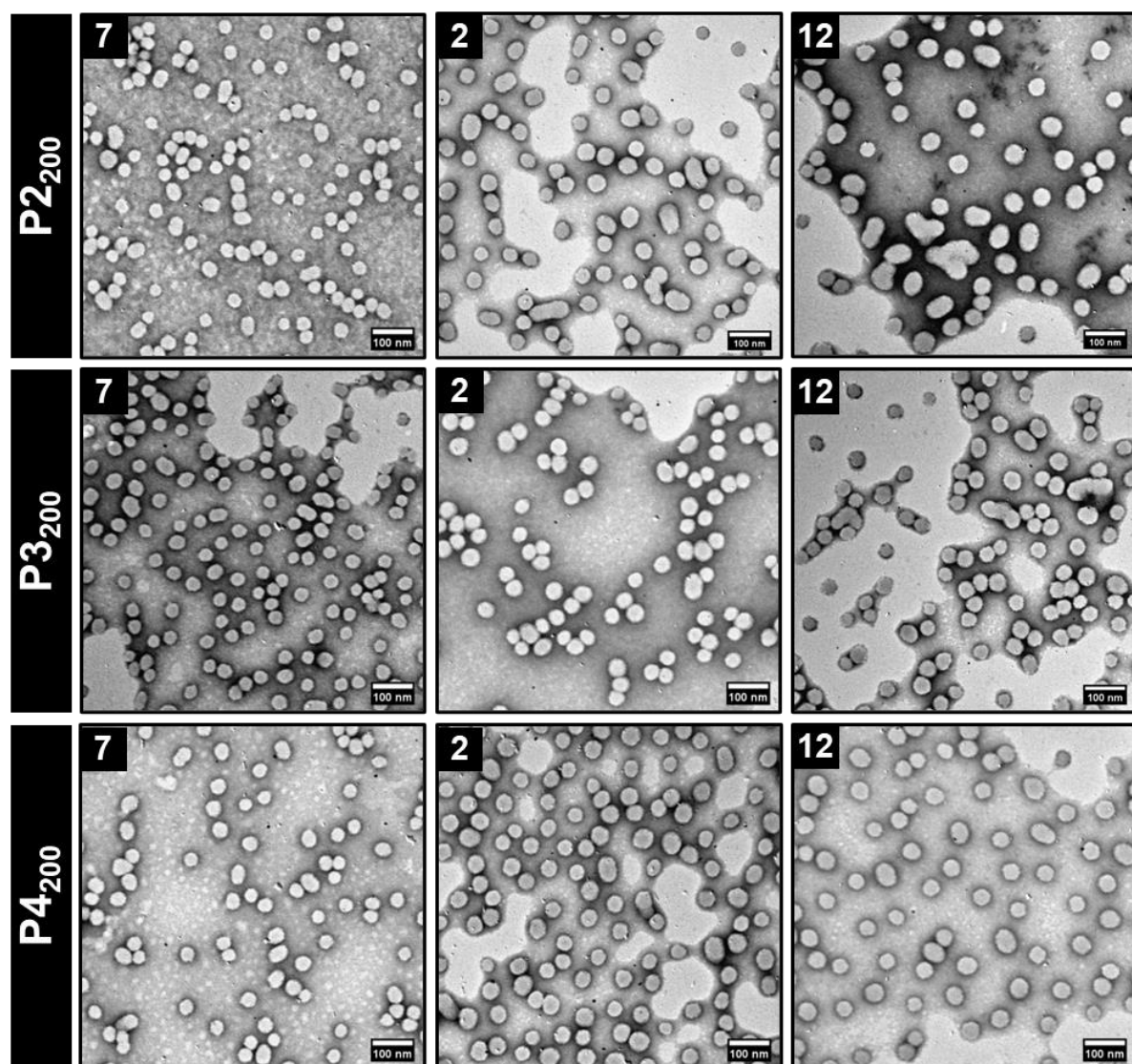


Figure S2.27. Supplementary dry-state TEM images of $P2_{200}$, $P3_{200}$ and $P4_{200}$ particles synthesised at pH 7, acidified to pH 2 and then basified to pH 12. Scale bar = 100 nm.

This process was repeated in the reverse order, first increasing the pH to 12 by addition of base, followed by a switch to pH 2.

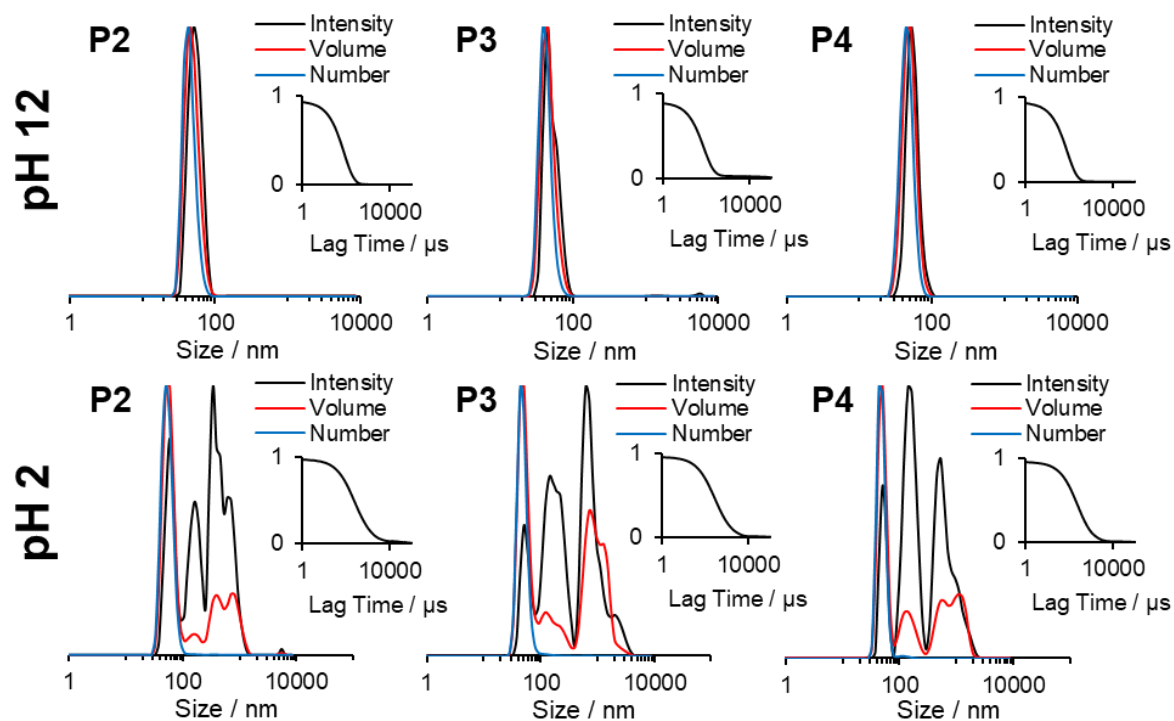


Figure S2.28. Normalised DLS analysis of **P2₂₀₀**, **P3₂₀₀**, and **P4₂₀₀** particles basified to pH 12 and then switched to pH 2.

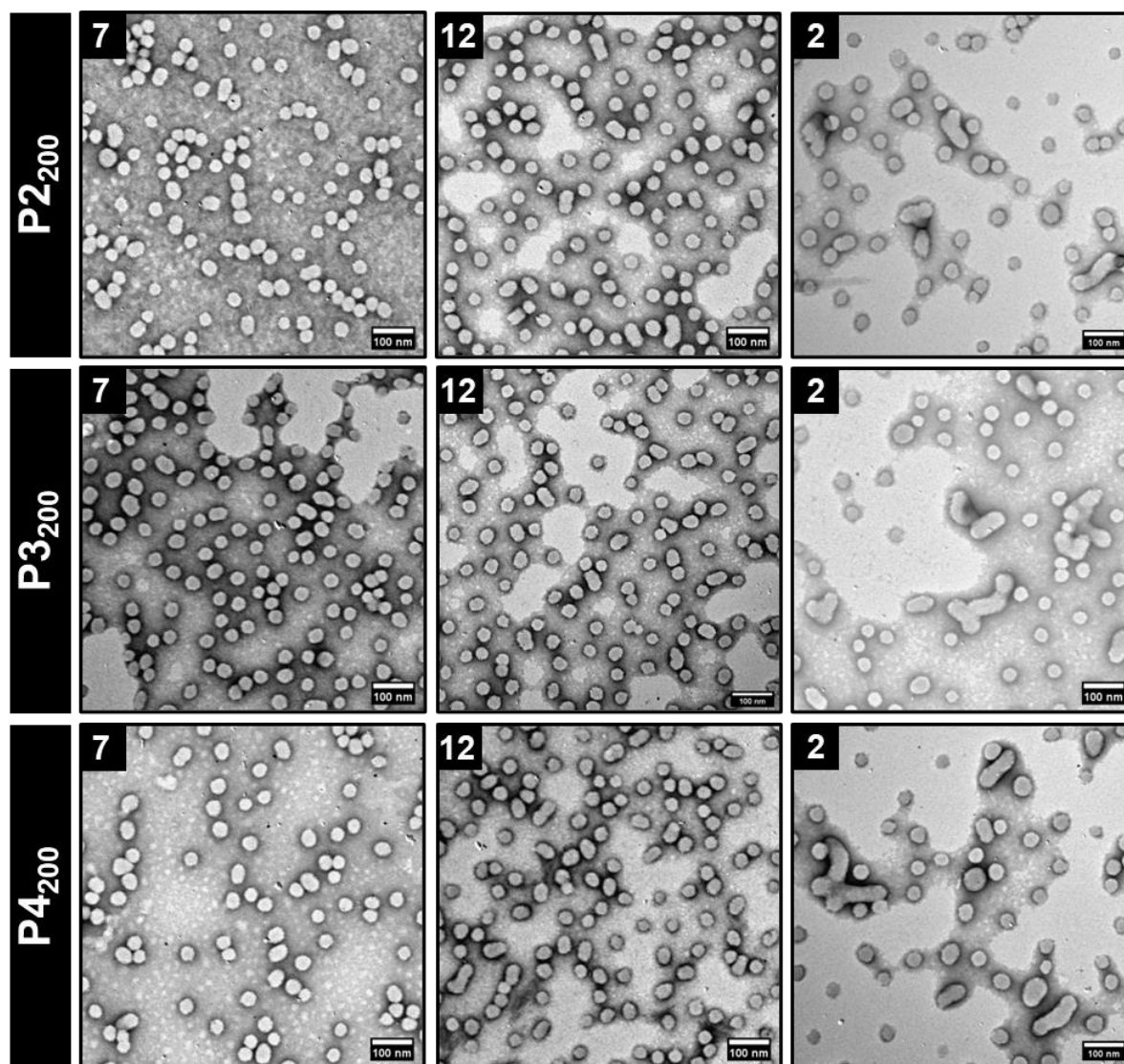


Figure S2.29. Supplementary dry-state TEM images of $P2_{200}$, $P3_{200}$ and $P4_{200}$ particles synthesised at pH 7, basified to pH 12 and then acidified to pH 2. Scale bar = 100 nm.

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Chapter 3. Investigating Charge-Mediated Fusion of Polymersomes Synthesised *via* Aqueous Ring-Opening Metathesis Polymerisation Induced Self-Assembly

3.1 Abstract

Membrane fusion in biology is a high selective and efficient process. This is achieved by several biological systems acting in unison to control the orientation and direction of fusion. A key obstacle in biological membrane fusion is overcoming the large energetic barrier due to charge repulsion from approaching lipid headgroups. However, it has been demonstrated that electrostatic attraction between oppositely charged lipids can be utilised as a driving force in synthetic liposome fusion. Polymersomes, a class of synthetic vesicles composed of a polymer membrane, display many benefits over liposomes, such as enhanced chemical diversity and improved stability. However, polymersome fusion remains a largely unexplored process.

Here, we explore the charge-mediated fusion of polymersomes prepared by ring-opening metathesis polymerisation induced self-assembly (ROMPISA). Cationic, amine- and anionic, carboxylic acid-functionalised polymersomes are mixed, resulting in a range of nano-object morphologies which are characterised by DLS and dry-state TEM. Variation of the core block length demonstrates the ability to use electrostatic attraction as a driving force for polymersome fusion. In addition, a size selectivity during charge-mediated polymersome fusion is also indicated. The mode of fusion between different polymersomes in a model system is probed using Förster resonance energy transfer (FRET) analysis, indicating the presence of a hetero-fusion mechanism, whereby fusion occurs between the two different particles. These results expand the scope for synthetic systems to mimic the fusion behaviour observed in biology and demonstrate the efficacy of fluorescence techniques to probe fusion pathway mechanisms.

3.2 Introduction

Membrane fusion is a fundamental process in complex biological systems and can occur between a multitude of structures, including cells, intracellular compartments, and lipid bound structures such as viral particles (Figure 3.1).¹ To undergo fusion, the membranes must be brought into close proximity, overcoming the electrostatic repulsion between similarly charged head groups, before a local bilayer destabilisation induces fusion.

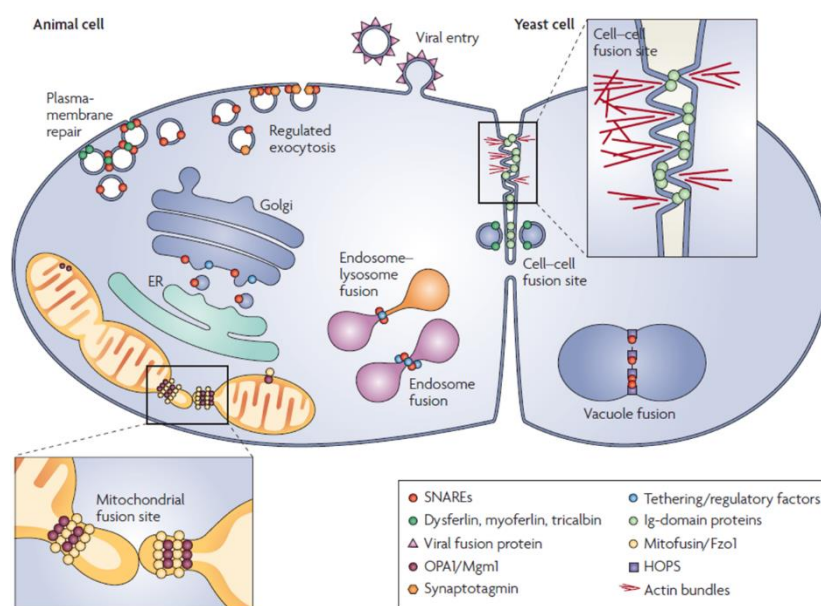


Figure 3.1. Mechanisms of membrane fusion in biological systems. Adapted from Martens *et al.*¹

The energy required to overcome the energy barrier for the fusion of biological membranes is provided by specialised fusion proteins, termed SNAREs. These molecules form complimentary interactions with one another and result in the formation of a highly stable *trans*-SNARE complex. The energy released upon complex formation is used to tether the two membranes together, known as a “docked” state.²

However, many SNARE complexes form with high stability that are not consistent with known trafficking pathways, suggesting the stability requirements of these molecules reduce their

specificity.³ It is well reported that only certain SNARE complexes are formed *in vivo*, thus this specificity must come by an alternative means.

Several sources for this precise specificity have been identified. For example, Rab proteins on a donor membrane can bind with effectors, proteins or lipids located on the acceptor membrane.⁴ This facilitates the recognition of a partner membrane by physical contact in a specific location, in a process termed “attachment”.⁶

In addition, the Sec1 protein family has been found to regulate the fusion process between the Golgi apparatus and plasma membrane through interactions with target-membrane SNAREs at the site of vesicle docking.^{6, 7} Recruitment of the Sec6/8 complex at the contact site forms a targeting patch for the delivery of transport vesicles to the basal-lateral membrane in epithelial cells.⁸

Taking inspiration from biological membrane fusion, there have been several reports of SNARE protein analogues utilised to mediate the fusion of synthetic lipid bilayers, through the incorporation of specific interactions.^{9, 10} Most widely investigated is the use of complimentary DNA strands on the exterior of the liposome which, when brought into close contact, form a double-strand helix that maintains the close distance between particles required for membrane fusion (Figure 3.2). This technique has been employed to demonstrate the sequential fusion of disparate compartments, stimuli-responsive fusion and specific control over the fusion site, often using Förster resonance energy transfer (FRET) and cascade reaction monitoring as analysis techniques.¹¹⁻¹⁴

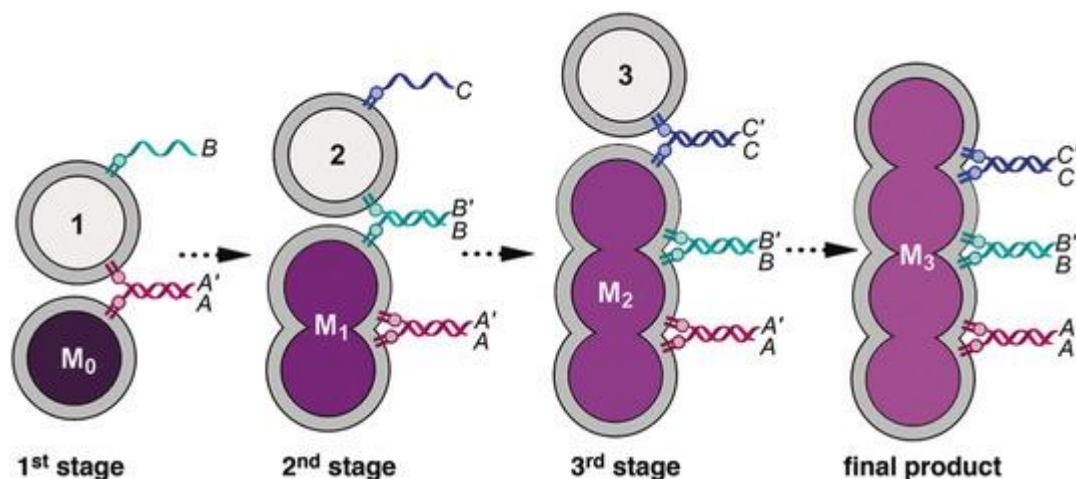


Figure 3.2 Schematic diagram of a three-stage liposome-fusion cascade encoded by three sets of complementary membrane-anchored DNA strands. Adapted from Löffler *et al.*¹¹

Several other methods have been implemented to mediate fusion in artificial liposomes, including peptides; boronic acid/*cis*-diol interactions; hydrogen bonding; click chemistry; inorganic nanoparticles; and reversible covalent bonding and caging.^{15–21} However, one of the simplest methods to mediate liposome fusion is through electrostatic interactions. Electrostatically induced, or charge-mediated, liposome fusion harnesses the attraction between oppositely charged particles to promote their selective aggregation and subsequent fusion.^{22, 23}

MacDonald and coworkers investigated the extent of lipid mixing between oppositely charged membranes as a function of surface charge, using content mixing assays to elucidate the extent of fusion.²⁴ They suggest that mutual neutralisation of the contacting monolayers reduces repulsion between head groups of the same type upon close contact. This process leads to deformations in the monolayers and allows the lipid tails to flow into contact through the openings. This causes contraction of the contacting surfaces and leads to surface fissures generating hemi-fused vesicles (merging of external monolayers with internal volumes

remaining separate). Finally, if membrane tension is large enough, the lysis tension will be overcome and lead to full bilayer fusion. Additional research in this area has also been reported.^{25, 26}

Polymersomes, synthetic vesicular structures formed of amphiphilic copolymers, are endowed with several benefits compared to liposomes, including higher stability and greater chemical versatility. However, owing to the high lysis tension of polymersomes, reports of their selective fusion behaviour are limited in comparison to liposomes.²⁷

The incorporation of fusogenic SNARE proteins into polymersomes formed of PDMS-*g*-PEO graft copolymers and their subsequent increased rate of fusion was demonstrated by Otrin and coworkers.²⁸ In addition, DNA-mediated aggregation of polymersomes has been explored, although fusion of the membranes was not reported.²⁹⁻³¹ However, inclusion of biological machinery (e.g. SNARE proteins) to promote the fusion of polymersomes is challenging due to the significant size mismatch between membrane proteins and polymersome membranes.³²

Recent work by Ivanov and coworkers has explored the utilisation of charge-mediated fusion in polymer/lipid hybrid membranes (Figure 3.3).³³ By incorporating cationic, anionic or zwitterionic lipids, the researchers displayed a high degree of membrane mixing between PDMS-*g*-PEO vesicles. As expected, the oppositely charged hybrid vesicle fused to the greatest degree, demonstrating that electrostatic attraction between polymersomes can be used as an energy source to drive membrane fusion.

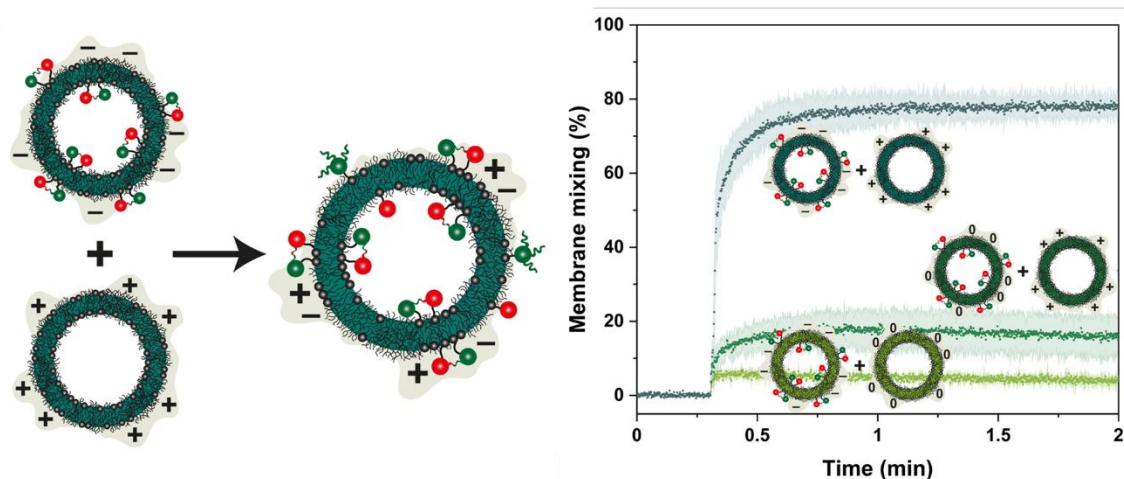


Figure 3.3. Charge-mediated membrane mixing of hybrid large unilamellar vesicles. Three combinations were tested: anionic/cationic, neutral/cationic, and anionic/neutral vesicles. Adapted from Marušič *et al.*³³

Consequently, it was hypothesised that, by incorporating oppositely charged functional groups within amphiphilic block copolymers, charge-mediated membrane fusion could be realised in a fully synthetic system (Figure 3.4).

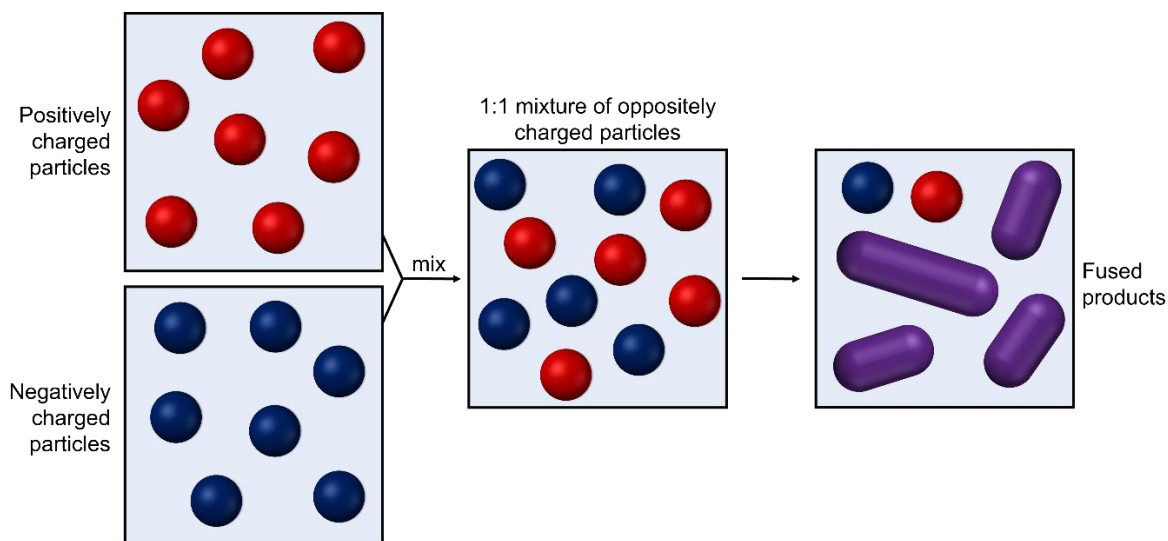


Figure 3.4. Schematic diagram of charge-mediated fusion between oppositely charged triblock polymersomes prepared *via* ROMPISA.

To that end, the synthesis of oppositely charged polymersomes by ROMPISA, and their subsequent charge-mediated fusion upon mixing, is reported. This is realised by incorporating either carboxylic acid- or amine-functionalised monomers as a charged block at the corona/core interface. The impact of increased membrane tension on the resulting fusion morphology is explored by varying the core DPs of the polymersomes.

In addition, the mode of fusion between different ROMPISA polymersomes is investigated. Using a model system, polymersomes are fluorescently labelled with either a pyrene- or naphthalimide-functionalised monomer. The efficiency of the FRET pair indicates the distance between the donor and acceptor dyes, facilitating distinction between a hetero- and homo-fusion process.

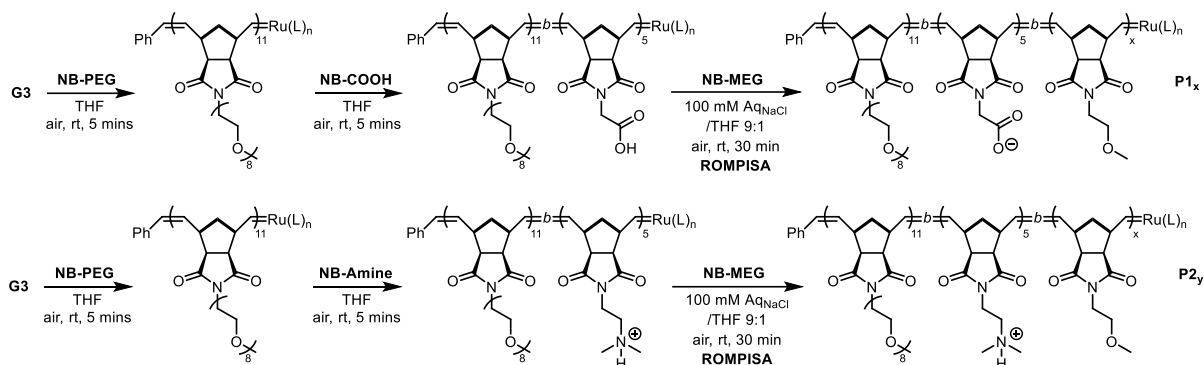
3.3 Results and Discussion

3.3.1 Synthesis of amine-containing triblock nano-objects *via* ROMPISA in salt solution

To mix oppositely charged particles and observe their resulting charge-mediated fusion, carboxylic acid- and amine-functionalised triblock nano-objects were synthesised *via* ROMPISA. Carboxylic acid groups typically have a pKa value below 5, while the value is above 9 for most simple, tertiary amines. To ensure both functionalities are in their charged state upon particle formation, the particles were synthesised at pH 7 using a solution of 100 mM NaCl in deionised water. By synthesising both sets of particles under the same conditions, we can keep the environment of the particles (e.g. pH) consistent upon mixing.

Carboxylic acid-functionalised triblock copolymer nano-objects (**P1_x**), comprised of a hydrophilic **NB-PEG** block, a pH-responsive **NB-COOH** block and **NB-MEG** as a core-forming block, were synthesised by ROMPISA and fully characterised by ¹H NMR spectroscopy, SEC, DLS and dry-state TEM, as described in Chapter 2, Section 2.3.1.3 (Scheme 3.1). Amine-containing triblock polymers, **P2_y**, were synthesised using the same procedure to that of **P1_x**, but with the substitution of the carboxylic acid-containing monomer (**NB-COOH**) for the previously reported amine-containing monomer, **NB-amine** (Scheme 3.1).

SEC analysis of **P2_y** showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.2$) for all core DPs (Table 3.1 and S3.1 and Figure 3.5).



Scheme 3.1. Synthetic route for the preparation of $P1_x$ and $P2_y$ triblock copolymers by ROMPISA at pH 7.

Table 3.1. Characterisation summary of $P2_y$.

Polymer	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\bar{D}_{GPC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
$P2_{100}$	29.8	30.0	1.21	37	0.23	29 ± 2
$P2_{200}$	51.9	50.0	1.12	44	0.17	34 ± 4
$P2_{300}$	74.0	65.0	1.11	52	0.18	37 ± 6

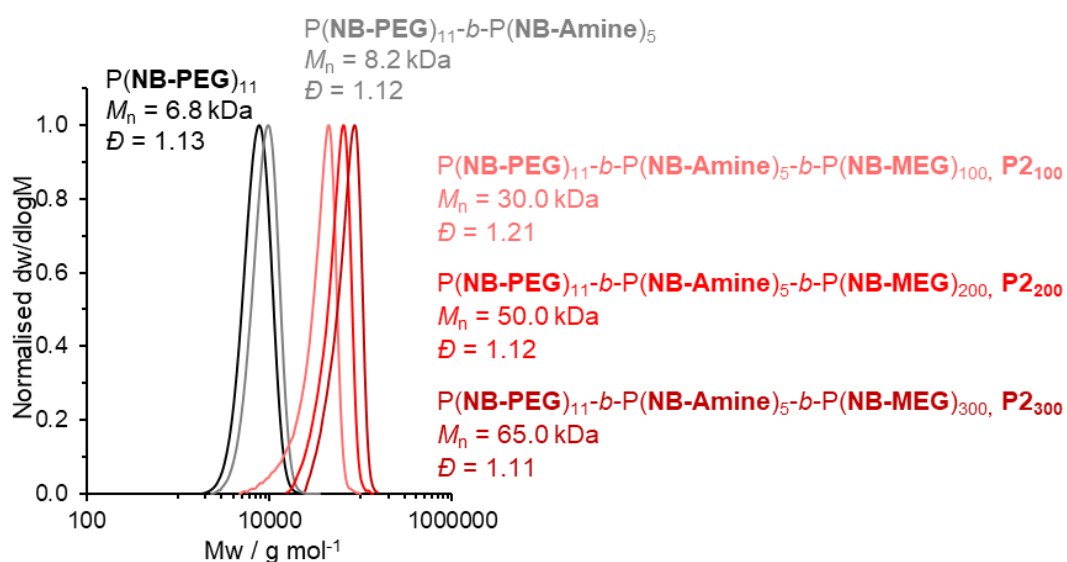


Figure 3.5. Normalised SEC traces (THF eluent, PMMA standards) of $P2_y$ and intermediate polymers.

DLS analysis showed narrowly dispersed nano-objects for **P2_y**, with average sizes of 37, 44 and 52 nm for DP 100, 200 and 300, respectively (Table 3.1 and Figure 3.6). **P2₁₀₀** and **P2₂₀₀** particles exhibited a spherical morphology by dry-state TEM with an average particle size of 29 and 34 nm, respectively (Table 3.1 and Figure 3.7, S3.8 and S3.9). **P2₃₀₀** particles were shown to spontaneously fuse to give short tubes with average lengths of 37 nm. This is likely due to the formation of particles with high membrane tension that overcame the threshold for spontaneous fusion despite the electrostatic repulsion induced by the positively charged amine groups. This result is consistent with a previous report detailing the synthesis of analogous particles at pH 2.³⁴

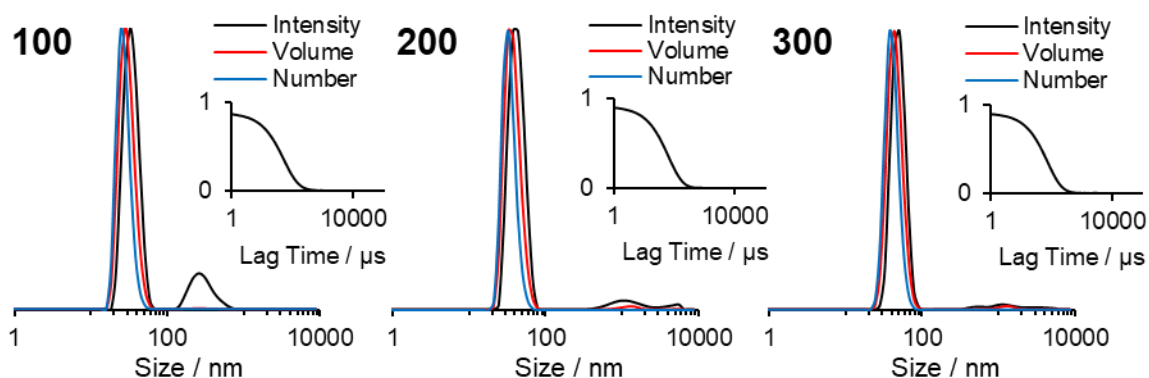


Figure 3.6. Normalised DLS analysis of **P2_y** particles: $y = 100, 200$, and 300 .

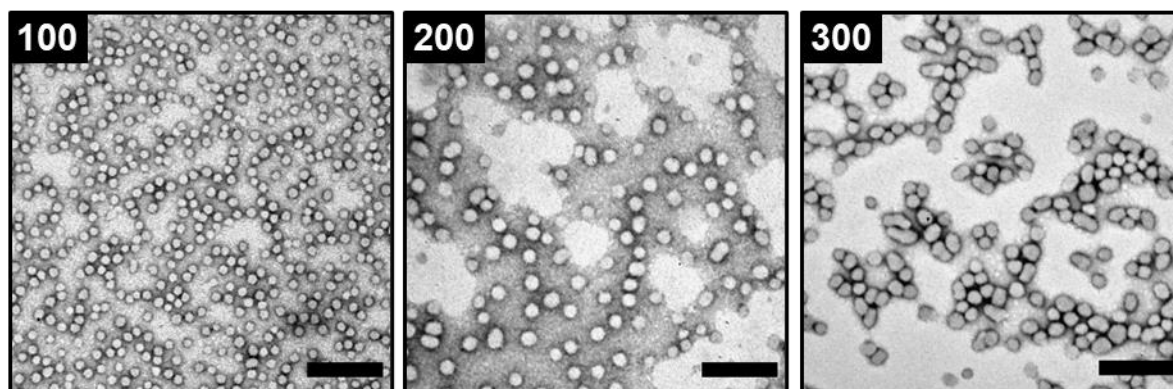


Figure 3.7. Dry-state TEM images of **P2_y** particles: $y = 100, 200$, and 300 . Scale bar = 200 nm.

3.3.2 Mixing oppositely charged polymersomes synthesised *via* ROMPISA

To explore the possible interactions between oppositely charged ROMPISA polymersomes, **P1_x** and **P2_y** were mixed in a vial to give a 1:1 mixture (**P1_x + P2_y**), which were then stirred for 30 mins. All solutions, except **P1₃₀₀ + P2₁₀₀**, showed an immediate increase in turbidity, suggesting an increase in particle size had occurred. The resulting solutions were then analysed by DLS and dry-state TEM analysis (Table 3.2).

Table 3.2. Characterisation summary for **P1_x + P2_y** mixtures.

Mixture	$Z_{\text{avg,DLS}} / \text{nm}$	PDI_{DLS}	$L_{\text{TEM}} / \text{nm}$
P1₁₀₀ + P2₁₀₀	88	0.18	77 ± 44
P1₁₀₀ + P2₂₀₀	94	0.15	66 ± 28
P1₁₀₀ + P2₃₀₀	85	0.12	39 ± 19
P1₂₀₀ + P2₁₀₀	91	0.22	47 ± 21
P1₂₀₀ + P2₂₀₀	179	0.22	127 ± 63
P1₂₀₀ + P2₃₀₀	176	0.20	67 ± 33
P1₃₀₀ + P2₁₀₀	61	0.28	45 ± 13
P1₃₀₀ + P2₂₀₀	95	0.16	57 ± 26
P1₃₀₀ + P2₃₀₀	140	0.14	127 ± 43

All mixtures displayed an increase in average particle size by DLS analysis compared to the original particle solutions. A unimodal distribution, with occasional shoulder peaks for x and y ≥ 200, was observed for all samples (Figure S3.10). Dry-state TEM analysis showed an increase in average particle size for all mixtures compared to their constituent particles (Figure 3.8,

S3.11 and S3.12). This implies that the two oppositely charged ROMPISA particles are interacting with each other when mixed. The only exception was $\mathbf{P1}_{300} + \mathbf{P2}_{100}$, where the average particle size (39 nm) was equivalent to that of $\mathbf{P2}_{100}$ particles (37 nm).

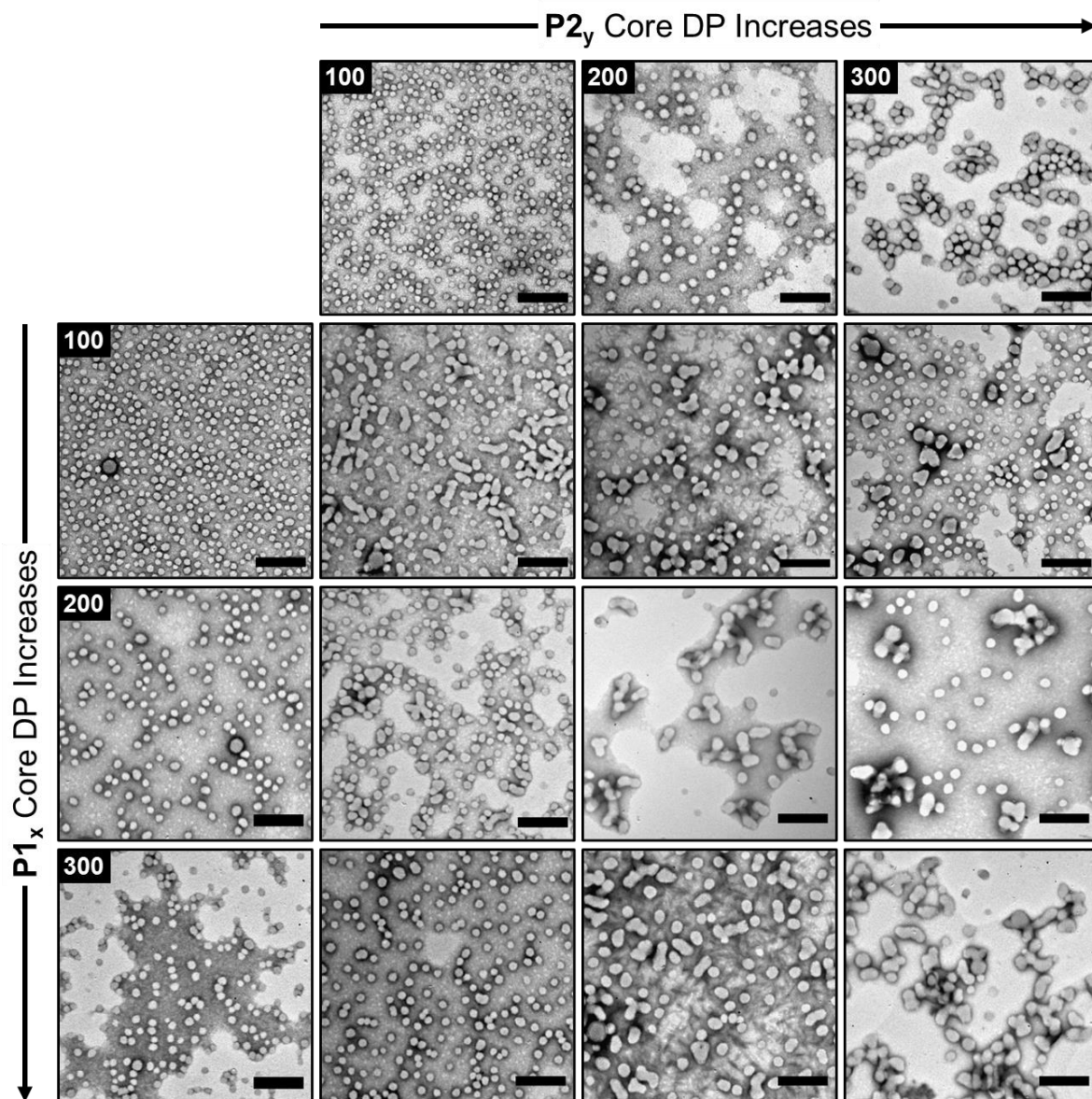


Figure 3.8. Dry-state TEM images of $\mathbf{P1}_x$ ($x = 100, 200$, and 300) and $\mathbf{P2}_y$ ($y = 100, 200$, and 300) particles and their corresponding mixtures ($\mathbf{P1}_x + \mathbf{P2}_y$) at the intersection. Scale bar = 200 nm.

All mixed samples containing $\mathbf{P1}_{100}$ particles ($\mathbf{P1}_{100} + \mathbf{P2}_y$) indicated a dual-morphology present when analysed by TEM, consisting of spherical particles and larger, fused structures. When

the DP for both particles was 100, fused tubes with occasional branching points were observed (Figure 3.8 and S3.11). This was surprising as both constituent particles are below the critical DP to fuse and suggests the electrostatic attraction between the oppositely charged particles may act as an energetic driving force to overcome the barrier to fusion.

In contrast, when **P2₂₀₀** particles were mixed with **P1₁₀₀**, irregular, “cluster” fused structures were formed, with a large central particle (similar in size to the original **P2₂₀₀** particle) surrounded by several smaller spherical particles, equivalent in size to **P1₁₀₀** particles. The electrostatic attraction between the two oppositely charged particles acts as a driving force for adhesion. However, the size disparity between the particles prevents chain rearrangement into a linear tube as this would require significant membrane deformation in the smaller particle, for which a significant energy barrier exists (Figure 3.9 A). Instead, there is only a small membrane restructuring of the larger particle, maintaining a high curvature across most of the particle. This leads to multiple fusion events across the particle surface, forming irregular cluster structures (Figure 3.9 B).

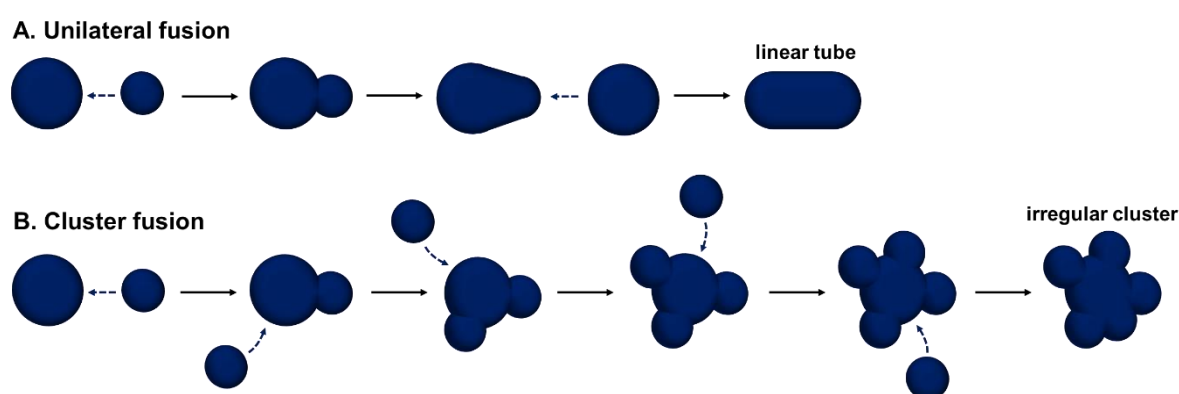


Figure 3.9. Suggested fusion mechanisms for (A) unilateral fusion and (B) cluster fusion.

TEM analysis of **P1₁₀₀** + **P2₃₀₀** showed a proportion of fused structures below 50%, indicating the anisotropic **P2₃₀₀** particles still maintain significant membrane tension to overcome the

barrier to successive fusion events, compared to the low membrane tension **P1₁₀₀**, which remain spherical (Figure 3.8 and S3.11).

P1₂₀₀ mixtures displayed a range of morphologies when mixed with **P2** particles of varying DP (Figure 3.8 and S3.11). Mixtures **P1₂₀₀ + P2₁₀₀** and **P1₂₀₀ + P2₃₀₀** produced solutions of mixed morphologies comprised predominantly of spherical nano-objects alongside a handful of larger structures. When **P2** DP 100, these larger structures are clearly fused tubes which occasionally branch off to form multi-arm structures, similar to the tetrapod nano-objects previously reported by Xiao *et al.*³⁵ However, when the DP is further increased to 300, the fusion process is less precise, resulting in irregular fused structures. In contrast, TEM analysis of **P1₂₀₀ + P2₂₀₀** displayed almost exclusively fused structures, forming frequently branched tubes.

Mixtures involving **P1₃₀₀** gave the largest variety in particle morphologies between different **P2** DPs. TEM analysis of **P1₃₀₀ + P2₁₀₀** displayed a population of almost entirely spherical nano-objects with an average size of 45 nm – larger than both constituent particles. This suggests that, although almost no fusion is occurring, the electrostatic attraction between the oppositely charged particles results in an increase in their diameter.

As seen for other **P1** particles, mixtures of **P1₃₀₀** and **P2** above DP 100 form fused structures. With **P2₂₀₀**, the resulting morphology is mixed between fused tubes and spherical nano-objects, whilst **P1₃₀₀ + P2₃₀₀** forms irregular fused structures with very few spherical particles still present. We attribute this irregularity to the high membrane tension instilled in both sets of particles at DP 300, resulting in a large excess of energy that drives fusion in an uncontrolled manner and leading to non-axial fusion events occurring.

Further conclusions can be drawn when comparing the average particle size to the core DPs for each constituent particle (Figure 3.10). Here, we can see there is a clear increase in the average particle size when the mixing particles have equivalent core DPs, with **P1₂₀₀ + P2₂₀₀** and **P1₃₀₀ + P2₃₀₀** mixtures resulting in an average particle size of 127 nm, whilst **P1₁₀₀ + P2₁₀₀** were measured at 77 nm. This difference is likely due to low membrane tension in both **P1₁₀₀** and **P2₁₀₀** particles, resulting in the electrostatic attraction between the two particles acting as the only driving force for fusion. In contrast, particles with core DPs ≥ 200 possess sufficient membrane tension to overcome the energetic barrier to fusion, as well as the additional driving force from electrostatic attraction. Further work will elucidate the possibility of any differences in the mechanism of electrostatically driven fusion compared to fusion driven by membrane tension release alone.

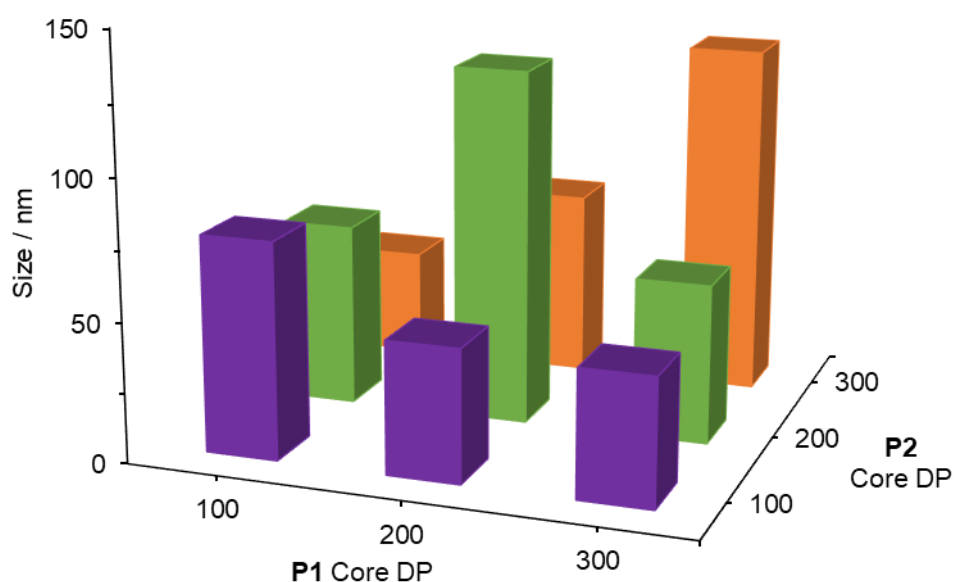


Figure 3.10. Average particles size, as determined by dry-state TEM, compared to the core DP for the constituent **P1_x** and **P2_y** particles.

As such, the relationship between average particle size and the equivalence of core DP between the two mixed particles, irrespective of the value, suggests a size selectivity is present during charge-mediated polymersome fusion. It was theorised that, as polymersomes undergo chain rearrangement, polymers of similar lengths are able to achieve a more ordered membrane packing (Figure 3.11). This allows for an alignment of the oppositely charged groups closer to one another, resulting in the formation of a structure lower in energy.

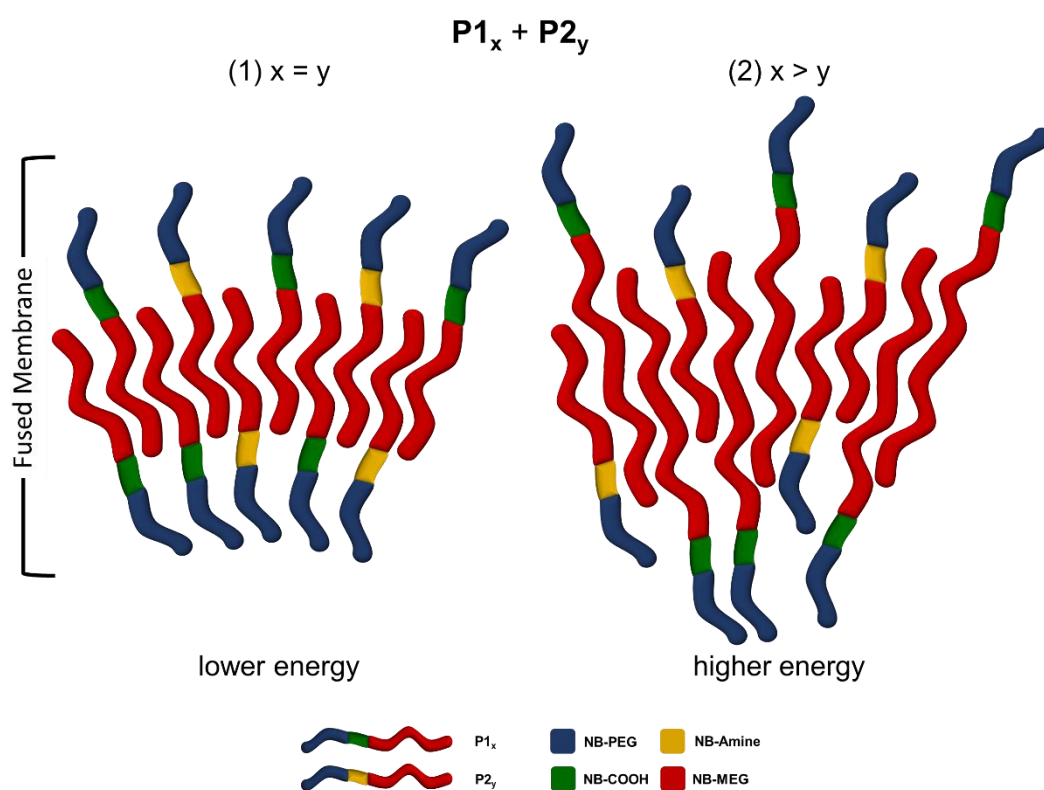


Figure 3.11. Suggested packing structures for fused membranes in $P1_x + P2_y$ mixtures with either (1) equivalent core DPs or (2) inequivalent core DPs between particles.

3.3.3 Determination of the Mode of Fusion (hetero- vs homo-fusion)

Mixing oppositely charged polymersomes results in various fused morphologies, depending on the DP of the constituent particles. However, amongst these fused products, unfused nano-objects remain, often in keeping with the size displayed by one of the original particles. Due to there being two different particles present in the mixture, there are two possible fusion pathways: (1) hetero-fusion, where fusion occurs between the two different particles, or (2) homo-fusion, in which fusion is only occurring between the same type of particle (Figure 3.12). Harnessing the ability to dictate which pathway is being followed would allow for further development of selective fusion between fully synthetic compartments, mirroring the adeptness demonstrated in biological systems.

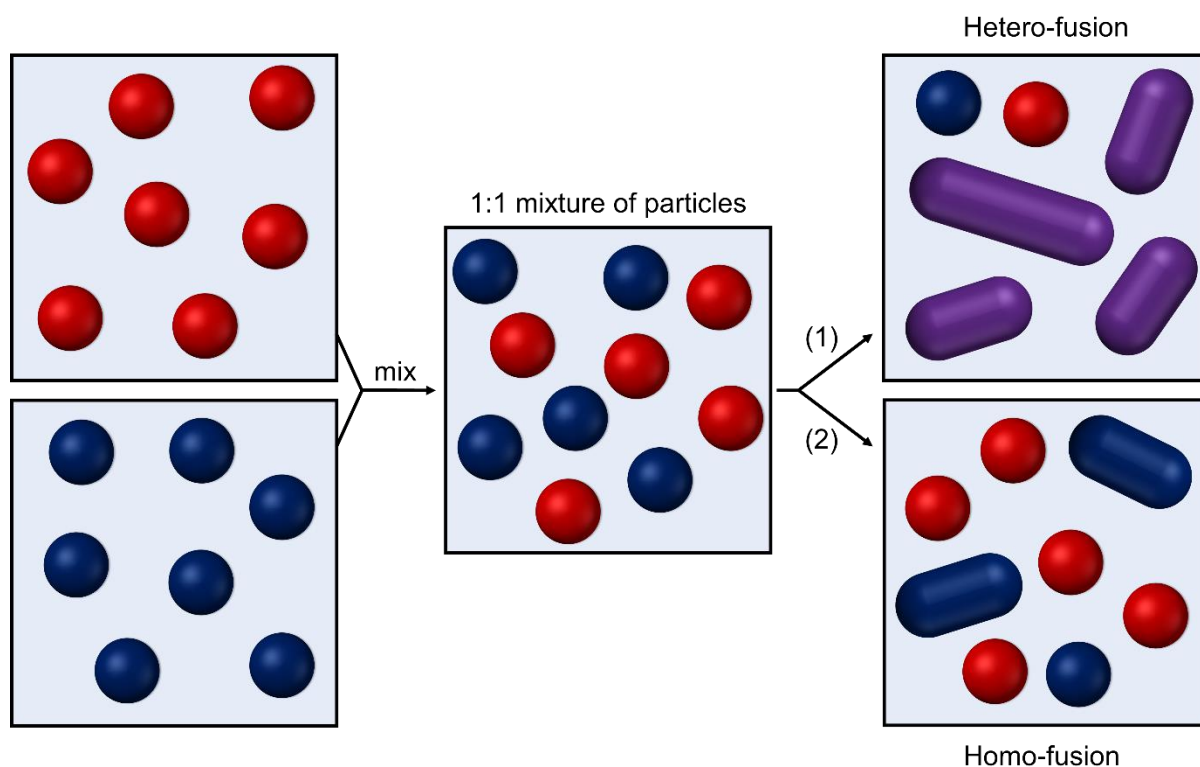


Figure 3.12. Proposed modes of fusion resulting from the mixing of two different particles.

Förster resonance energy transfer (FRET) is a process by which the excited state energy of one fluorescent dye is transferred non-radiatively to another dye.³⁶ Since the efficiency of FRET is dependent upon the distance between the donor and acceptor dyes, this technique can be used to determine the nature of the resultant fusion product upon mixing two different particles, one containing the donor dye and the other the acceptor.

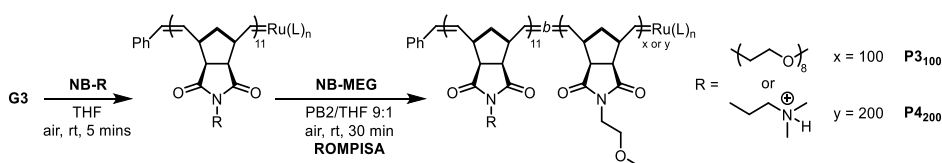
If homo-fusion occurs, the donor and acceptor dyes will not be brought into close proximity, meaning no energy transfer will occur between them and the fluorescence response of the system will not change. Alternatively, if hetero-fusion occurs, where there is fusion between the particles containing a donor and acceptor dye, energy transfer between the FRET pair would result in an increase in acceptor emission. FRET is a widely used technique for visualising morphological transitions in polymersomes, as well as the fusion of lipid and/or polymersome membranes.^{33, 37-41}

3.3.3.1 Synthesis and characterisation of diblock nano-objects synthesised *via* ROMPISA

To evaluate the mode of fusion between two different particles, initial experiments were conducted using a simplified system which removed the complexity of multiple charges within the system. For this, the amphiphilic diblock copolymers, **P3_x**, **P(NB-PEG)₁₁-b-P(NB-MEG)_x**, and **P4_y**, **P(NB-Amine)₁₁-b-P(NB-MEG)_y**, containing a corona formed from discrete blocks of either **NB-PEG** or **NB-Amine** and a core of **NB-MEG** (DP = x or y) were accessed *via* ROMPISA (Scheme 3.2). **P3_x** polymersomes have been shown to spontaneously fuse during polymerisation at a DP ≥ 140, and so a DP of 100 was targeted (**P3₁₀₀**) to ensure a spherical morphology prior to mixing.⁴²

Similarly, **P4_y** are limited to spontaneous fusion only above DP 300 when synthesised at pH 2 due to the electrostatic repulsion between the charged particles. However, it has been demonstrated that amine-functionalised polymersomes can fuse when this charge is screened (e.g. by NaCl), as long as the DP is above 180. As such, a DP of 200 was targeted (**P4₂₀₀**) to produce nano-objects with a spherical morphology that contain significant membrane tension to drive fusion when released.

As previously reported, the corona was first synthesised to produce nano-objects by reacting the **G3** initiator with the hydrophilic monomer, **NB-PEG** or **NB-Amine**, in THF.⁴³ The resulting water-soluble macroinitiator was then chain-extended upon addition of **NB-MEG** dissolved in 100 mM PB2 to give a final solvent composition of THF/PB2 of 1:9 v/v.

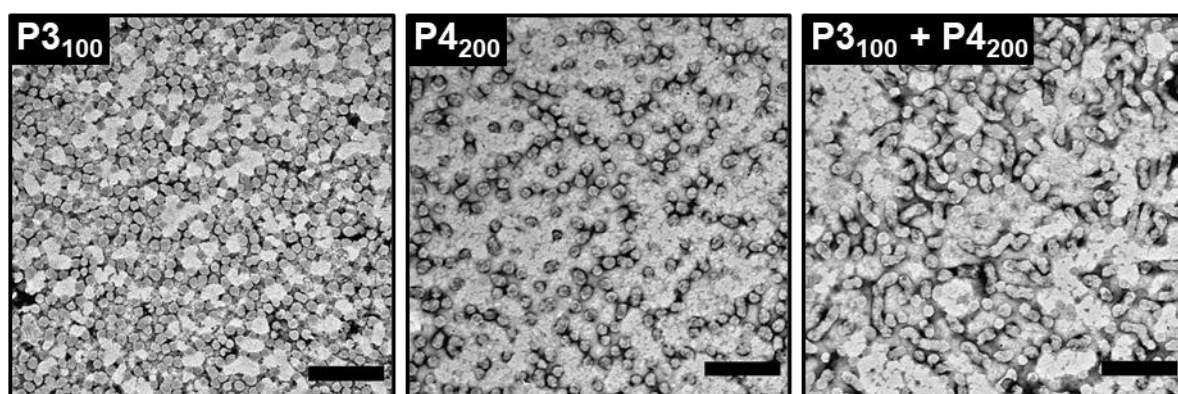


Scheme 3.2. Synthetic route for the preparation of **P3₁₀₀** and **P4₂₀₀** block copolymers by ROMPISA at pH 2.

SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} = 1.1$) for all core DPs (Table 3.3 and S3.2 and Figure S3.13). The nano-objects formed during the ROMPISA process were then analysed by DLS and displayed average diameters of 49 and 53 nm for **P3₁₀₀** and **P4₂₀₀**, respectively (Table 3.3 and Figure S3.14). In line with previous reports, TEM analysis showed spherical polymersomes for both **P3₁₀₀** and **P4₂₀₀** (Table 3.3 and Figure 3.13 and S3.15).

Table 3.3. Characterisation summary of **P3₁₀₀**, **P4₂₀₀**, and **P3₁₀₀ + P4₂₀₀**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\bar{D}_{GPC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
P3₁₀₀	28.7	30.9	1.09	49	0.20	29 ± 2
P4₂₀₀	47.6	48.3	1.11	53	0.17	21 ± 2
P3₁₀₀ + P4₂₀₀	N/A	N/A	N/A	65	0.16	64 ± 18

**Figure 3.13.** Dry-state TEM images of **P3₁₀₀**, **P4₂₀₀**, and **P3₁₀₀ + P4₂₀₀**. Scale bar = 200 nm.

3.3.3.2 Mixing of diblock nano-objects

In line with data published in a previous study, **P4₂₀₀** nano-objects do not spontaneously fuse during polymerisation due to the electrostatic repulsion between positively charged particles.⁴² However, they do contain membrane tension above the fusion threshold that would act as a thermodynamic driving force for them to fuse when the electrostatic repulsion is removed (e.g. by a pH switch).

However, similarly to the mixed formulations discussed previously, it was theorised that mixing **P3₁₀₀** and **P4₂₀₀** particles would allow the release of the membrane tension within the amine-containing particles, resulting in the fusion of polymersomes. To test this hypothesis, **P3₁₀₀** and **P4₂₀₀** were mixed in a vial to give a 1:1 mixture (**P3₁₀₀ + P4₂₀₀**) which was then stirred

for 30 mins. The solution showed an immediate increase in turbidity, suggesting an increase in particle size had occurred. DLS analysis of the resulting mixture showed an average particle size of 67 nm, whilst TEM analysis showed the change in morphology from spherical nano-objects to fused tubes with average length 64 nm (Table 3.3 and Figure 3.13, S3.14 and S3.15).

3.3.3.3 Synthesis and characterisation of fluorescent monomers for use in ROMPISA

To investigate the nature of fusion between these two disparate particles, fluorescent monomers that can combine to form a FRET pair were synthesised. It has previously been reported that pyrene can act as a fluorescent donor when in close proximity to naphthalimide based dyes, which then acts as an acceptor, thus forming a FRET pair.

As such, *exo*-norbornene pyrene (**NB-Pyr**) and *exo*-norbornene morpholine naphthalimide (**NB-NI**) were synthesised (Figure 3.14). UV-vis and fluorescence spectroscopy show good overlap between the **NB-Pyr** emission spectrum and the **NB-NI** excitation spectrum, highlighting their potential in forming a FRET pair (Figure 3.15).

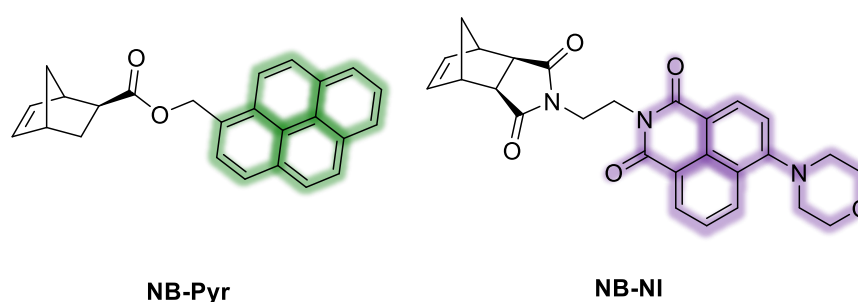


Figure 3.14. Chemical structures for fluorescent ROMPISA monomers **NB-Pyr** and **NB-NI**.

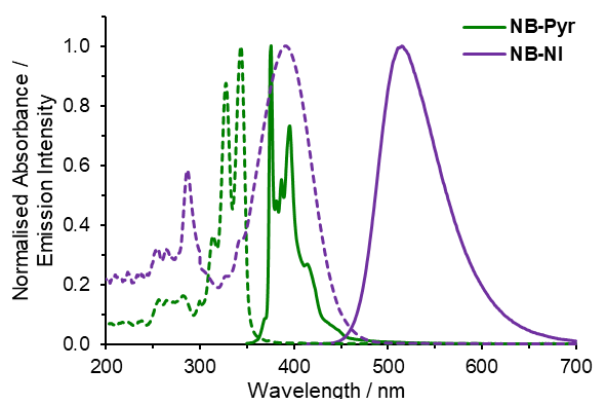


Figure 3.15. Normalised UV-vis absorption spectra (dotted lines) of **NB-Pyr** (green) and **NB-NI** (purple).

Normalised fluorescence emission spectra (solid lines) of **NB-Pyr** ($\lambda_{\text{Ex}} = 347$ nm, green) and **NB-NI** ($\lambda_{\text{Ex}} = 405$ nm, purple).

3.3.3.4 Synthesis and characterisation of fluorescently labelled triblock nano-objects *via* ROMPISA

Four fluorescently labelled polymersomes were synthesised by adapting a previously reported method for triblock polymersome synthesis.³⁴ For **P3-Pyr**₁₀₀, the corona was synthesised by reacting the **G3** initiator with the hydrophilic monomers, first **NB-PEG** and then **NB-Pyr** in tetrahydrofuran (THF). The resulting water-soluble macroinitiator was then chain-extended upon addition of **NB-MEG** dissolved in 100 mM PB2 to give a final solvent composition of THF/PB2 of 1:9 v/v. This process was then repeated using **NB-Amine** as a corona monomer and **NB-NI** as the fluorescent dye to produce **P4-NI**_{100/200}. Lastly, an amine-functionalised polymersome containing both **NB-Pyr** and **NB-NI** was synthesised as a control sample (**P4-Pyr-NI**₂₀₀) (Figure 3.16). The properties of the fluorescent particles synthesised are detailed in Table 3.4.

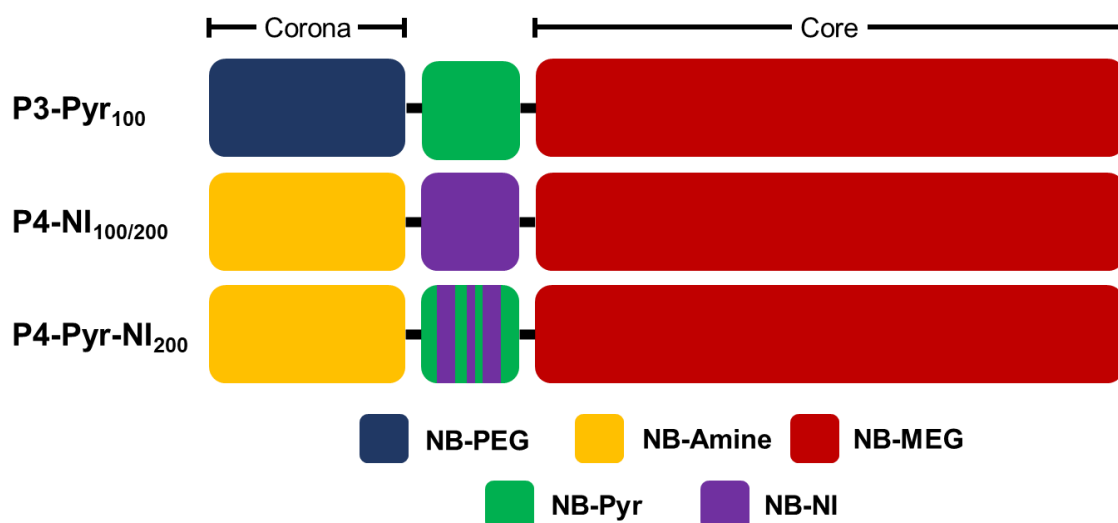


Figure 3.16. Schematic diagram showing the different monomers and microstructures targeted for the polymers in each fluorescently labelled particle.

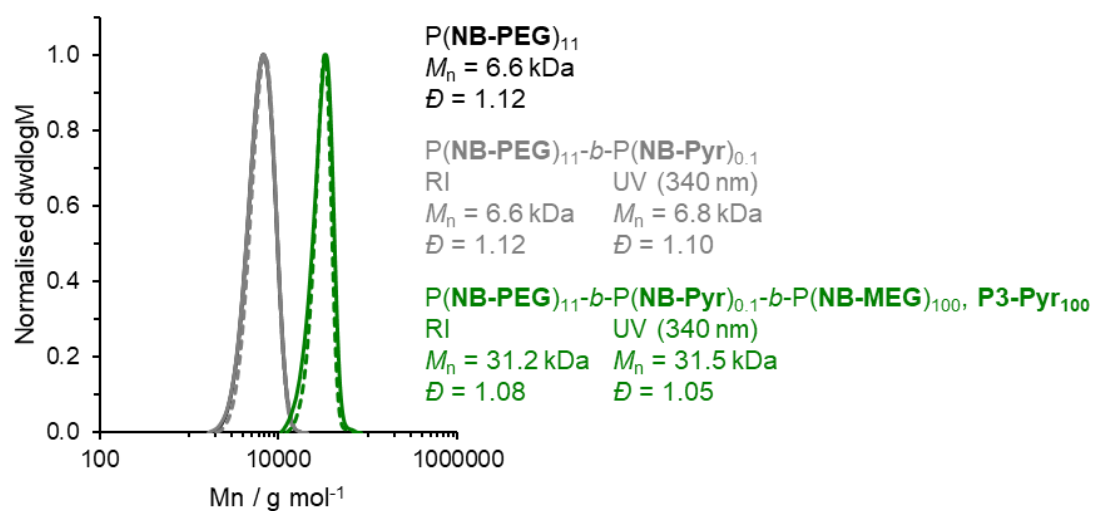
Table 3.4. Property information for fluorescent ROMPISA particles and the relevant control particles.

Polymer	Corona	Core DP	Fluorescent Dye
P3-Pyr₁₀₀	NB-PEG	100	NB-Pyr
P4-NI₁₀₀	NB-Amine	100	NB-NI
P4-NI₂₀₀	NB-Amine	200	NB-NI
P4-Pyr-NI₂₀₀	NB-Amine	200	NB-Pyr and NB-NI

An increase in turbidity was observed for all solutions. SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} = 1.1$) for all core DPs (Table 3.5 and S3.2 and Figure 3.17, 3.18, 3.19 and 3.20). Additionally, an overlay of data collected from the UV absorbance and refractive index detectors shows the successful incorporation of the fluorescent dye(s) in each polymer. DLS and TEM analysis shows no change in particle size or morphology with the inclusion of either fluorescent dye (Table 3.5 and Figure S3.14, 3.21 and S3.15).

Table 3.5. Characterisation summary of **P3-Pyr₁₀₀**, **P4-NI₁₀₀**, **P4-NI₂₀₀** and **P4-Pyr-NI₂₀₀**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\bar{D}_{GPC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
P3-Pyr₁₀₀	28.7	31.2	1.08	57	0.33	28 ± 2
P4-NI₁₀₀	25.5	28.0	1.09	92	0.30	12 ± 1
P4-NI₂₀₀	47.7	48.0	1.09	116	0.24	30 ± 3
P4-Pyr-NI₂₀₀	47.7	48.2	1.10	103	0.21	23 ± 2

**Figure 3.17.** Normalised SEC traces (THF eluent, PMMA standards) of **P3-Pyr₁₀₀** and intermediate polymers. RI (solid line) and UV 340 (dotted line).

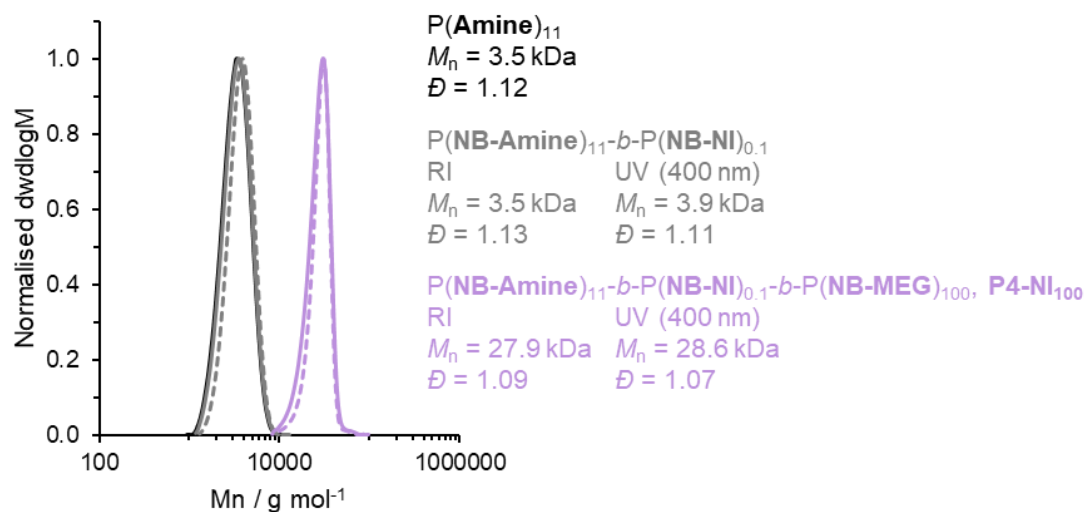


Figure 3.18. Normalised SEC traces (THF eluent, PMMA standards) of **P4-NI₁₀₀** and intermediate polymers. RI (solid line) and UV 400 nm (dashed line).

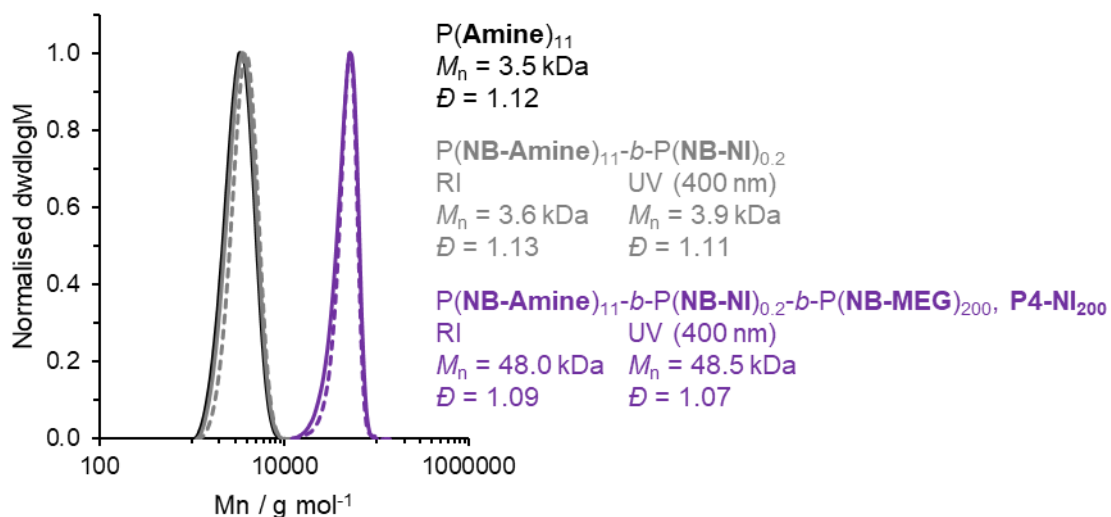


Figure 3.19. Normalised SEC traces (THF eluent, PMMA standards) of **P4-NI₂₀₀** and intermediate polymers. RI (solid line) and UV 400 nm (dashed line).

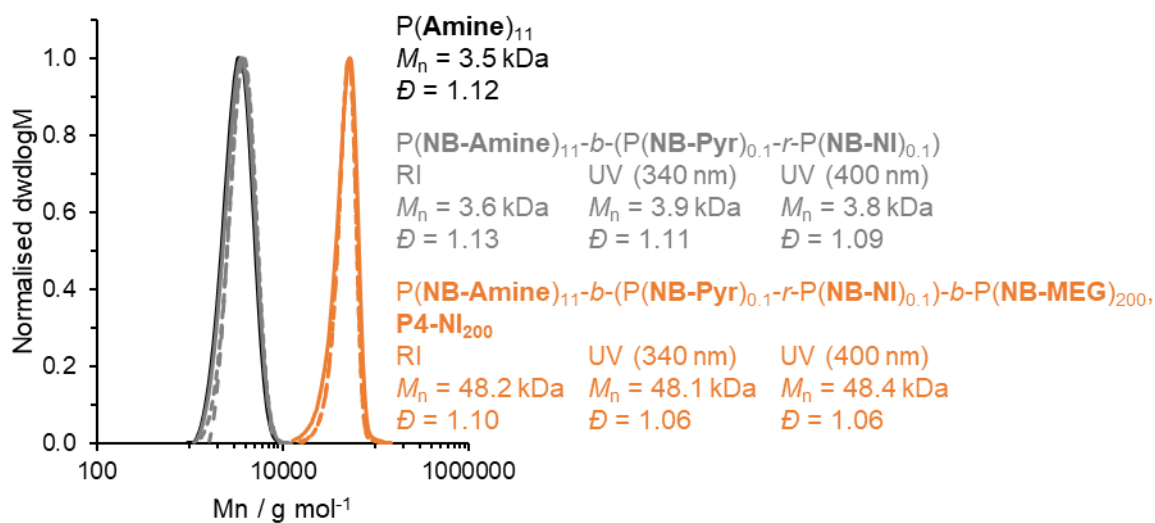


Figure 3.20. Normalised SEC traces (THF eluent, PMMA standards) of **P4-Pyr-NI₂₀₀** and intermediate polymers. RI (solid line), UV 340 (dotted line) and UV 400 nm (dashed line).

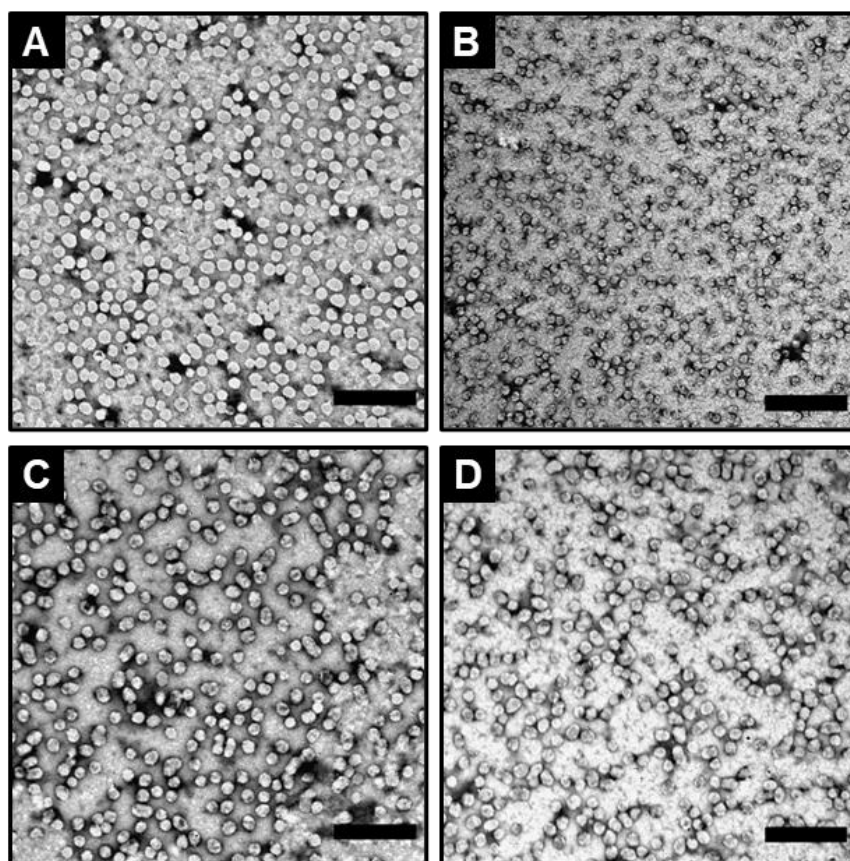


Figure 3.21. Dry-state TEM images of (A) **P3-Pyr₁₀₀**, (B) **P4-NI₁₀₀**, (C) **P4-NI₂₀₀**, (D) **P4-Pyr-NI₂₀₀**. Scale bar = 200 nm.

Fluorescence emission spectra were obtained for each particle at either $\lambda_{\text{Ex}} = 347$, $\lambda_{\text{Ex}} = 405$ nm or both (Figure 3.22). There was no change in the maximum λ_{Em} for **NB-Pyr** when incorporated into the particles (Figure 3.22, A). However, the maximum wavelength of emission for the **NB-NI** monomer increases by around 5-10 nm when included in a polymer (Figure 3.22, B and C). This could either be due to the change in solvent from 100% THF to a 9:1 PB2:THF mixture, and thus the impact of polarity, pH and hydrogen bonding must also be mentioned.⁴⁴ Alternatively, the change in the λ_{Em} maximum may also be caused by the presence of a closely neighbouring amine group, which has previously been reported to impact fluorescence when close to a dye molecule.⁴⁵

Furthermore, the emission spectra at $\lambda_{\text{Ex}} = 347$ and 405 nm for **P4-Pyr-NI₂₀₀** are identical to that of the single-dye particles, demonstrating that the presence of both dyes within a single particle does not affect their fluorescence properties (Figure 3.22, D). In addition, due to the close proximity of the pyrene and naphthalimide dyes, FRET is also observed when **P4-Pyr-NI₂₀₀** is excited at 347 nm, with a fluorescence emission intensity maximum around 75% of when the particle is excited at 405 nm (Figure 3.22, D, orange line).

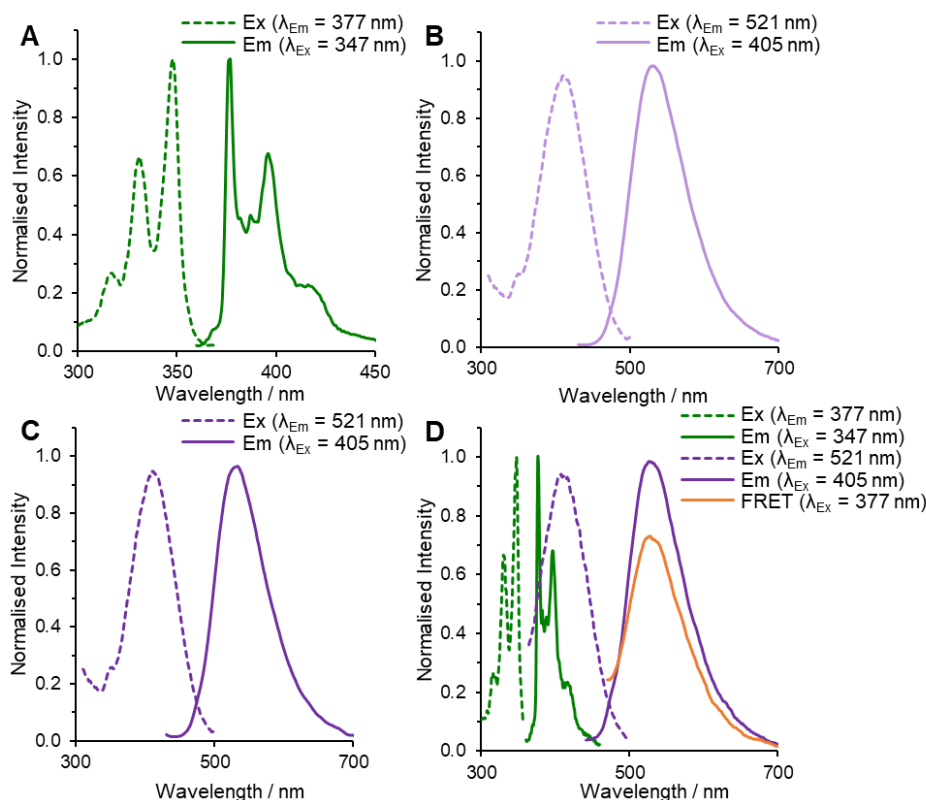


Figure 3.22. Normalised fluorescence excitation (dotted lines) and emission (solid lines) spectra of (A) **P3-Pyr**₁₀₀, (B) **P4-NI**₁₀₀, (C) **P4-NI**₂₀₀ and (D) **P4-Pyr-NI**₂₀₀. Green lines refer to **NB-Pyr** fluorescence ($\lambda_{\text{Ex}} = 347$, $\lambda_{\text{Em}} = 377$ nm), purple lines refer to **NB-NI** fluorescence ($\lambda_{\text{Ex}} = 405$, $\lambda_{\text{Em}} = 512$ nm), orange lines refer to **NB-NI** fluorescence ($\lambda_{\text{Ex}} = 347$, $\lambda_{\text{Em}} = 512$ nm).

3.3.3.5 Mixing of fluorescently labelled diblock nano-objects

As the addition of a small amount of fluorescent dye(s) into each type of particle did not affect the size or morphology, it was then decided to establish if they still maintained the required membrane tension to drive fusion upon mixing.

As such, 200 μL of **P3-Pyr**₁₀₀ (1 wt%, PB2 + 10% THF) was added to **P4-NI**₂₀₀ (200 μL , 1 wt%, PB2 + 10% THF) in a vial to give a 1:1 mixture (**P3-Pyr**₁₀₀ + **P4-NI**₂₀₀) which was then stirred for 30 minutes. In addition, several control mixing experiments were performed, as detailed in Table S3.3. All solutions, except **P3-Pyr**₁₀₀ + **P4-NI**₁₀₀, showed an immediate increase in turbidity,

suggesting an increase in particle size had occurred. The resulting solutions were analysed by DLS and dry-state TEM (Table 3.7).

DLS analysis showed unimodal distributions for all mixed solutions with an average mean diameter between 60-110 nm (Figure S3.16). Dry-state TEM analysis showed only spherical nano-objects for **P3-Pyr₁₀₀ + P4-NI₁₀₀** with an average particle size of 14 nm (Figure 3.23 and S3.17). However, fused tubes were observed for all other mixing experiments, with average particle lengths for both fluorescent and non-fluorescent nano-objects between 54-88 nm (Figure 3.23 and S3.17). Therefore, it can be concluded that adding a fluorescent dye does not impact the fusion process when **P3₁₀₀** and **P4₂₀₀** particles are mixed in a 1:1 ratio.

Table 3.6. Characterisation summary for **P3 + P4** mixtures.

Sample	$Z_{avg,DLS}$ /nm	PDI_{DLS}	L_{TEM} /nm
P3₁₀₀ + P4₂₀₀	65	0.16	64 ± 18
P3₁₀₀ + P4-NI₂₀₀	67	0.13	79 ± 25
P3₁₀₀ + P4-Pyr-NI₂₀₀	81	0.20	73 ± 22
P3-Pyr₁₀₀ + P4₂₀₀	68	0.19	88 ± 23
P3-Pyr₁₀₀ + P4-NI₁₀₀	109	0.18	14 ± 2
P3-Pyr₁₀₀ + P4-NI₂₀₀	67	0.19	54 ± 11

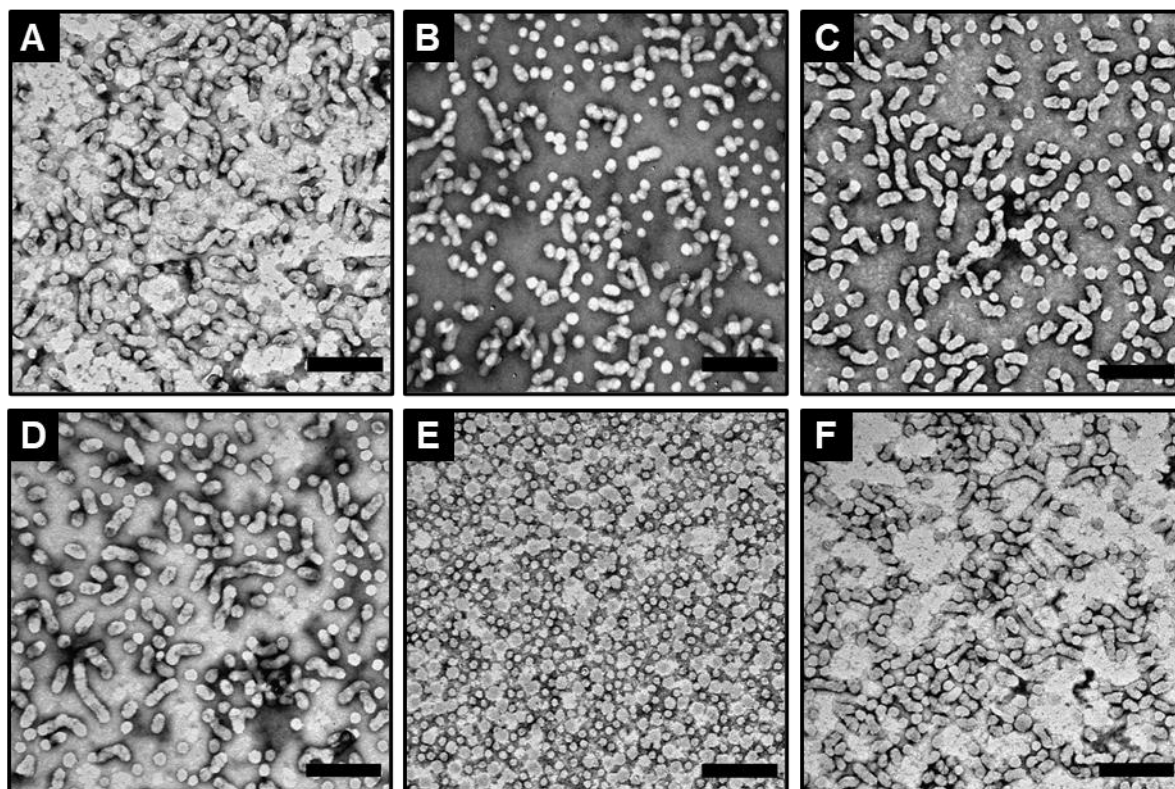


Figure 3.23. Dry-state TEM images of (A) $\text{P3}_{100} + \text{P4}_{200}$, (B) $\text{P3}_{100} + \text{P4-NI}_{200}$, (C) $\text{P3}_{100} + \text{P4-Pyr-NI}_{200}$, (D) $\text{P3-Pyr}_{100} + \text{P4}_{200}$, (E) $\text{P3-Pyr}_{100} + \text{P4-NI}_{100}$, (F) $\text{P3-Pyr}_{100} + \text{P4-NI}_{200}$. Scale bar = 200 nm.

Fluorescence emission spectra were obtained for each mixture at either $\lambda_{\text{Ex}} = 347$ ($\text{P3-Pyr}_{100} + \text{P4}_{200}$, $\text{P3-Pyr}_{100} + \text{P4-NI}_{100}$ and $\text{P3-Pyr}_{100} + \text{P4-NI}_{200}$) or $\lambda_{\text{Ex}} = 405$ nm ($\text{P3}_{100} + \text{P4-NI}_{200}$, $\text{P3}_{100} + \text{P4-Pyr-NI}_{200}$, $\text{P3-Pyr}_{100} + \text{P4-NI}_{100}$ and $\text{P3-Pyr}_{100} + \text{P4-NI}_{200}$) (Figure 3.24 and S3.14). Emission data from $\text{P3}_{100} + \text{P4-NI}_{200}$, $\text{P3}_{100} + \text{P4-Pyr-NI}_{200}$ and $\text{P3-Pyr}_{100} + \text{P4}_{200}$ confirm the fluorescence response of each individual dye, as well as a combination of both, are not affected by the change in morphology from spherical nano-objects to fused tubes (Figure S3.18).

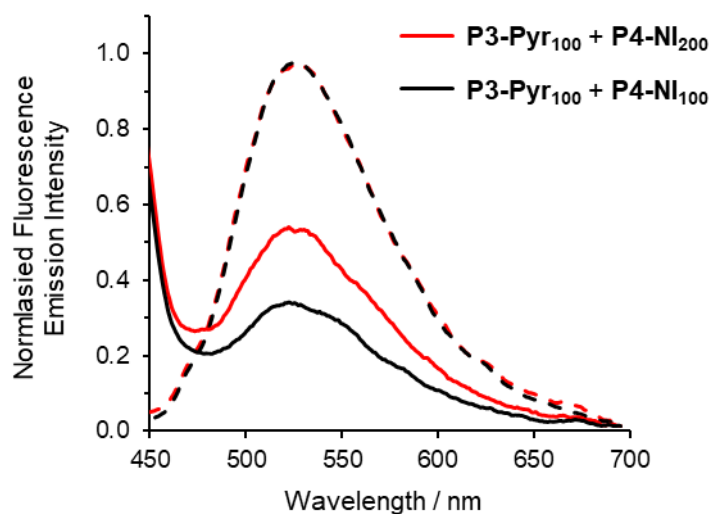


Figure 3.24. Normalised fluorescence emission spectra of **P3-Pyr₁₀₀ + P4-NI₁₀₀** (black line) and **P3-Pyr₁₀₀ + P4-NI₂₀₀** (red line). Fluorescence emission at $\lambda_{Ex} = 347$ nm (solid lines) was normalised to the emission intensity at $\lambda_{Ex} = 405$ nm (dashed lines).

At $\lambda_{Ex} = 347$ nm, **P3-Pyr₁₀₀ + P4-NI₁₀₀** and **P3-Pyr₁₀₀ + P4-NI₂₀₀** both displayed a fluorescence emission peak around 520 nm, synonymous with **NB-NI** emission and confirming FRET is taking place (Figure 3.24). Due to the variation in concentration of the acceptor group within a sample, the fluorescence emission intensity measured was normalised by the following equation:

$$\% FRET = \frac{I_{Em}(A)_{\lambda_{Ex} = \lambda_{Ex}^{max}(D)}}{I_{Em}(A)_{\lambda_{Ex} = \lambda_{Ex}^{max}(A)}} \times 100\% \quad 3.1$$

where $I_{Em}(A)_{\lambda_{Ex} = \lambda_{Ex}^{max}(D)}$ is the emission intensity of the acceptor when excited at the maximum excitation wavelength for the donor and $I_{Em}(A)_{\lambda_{Ex} = \lambda_{Ex}^{max}(A)}$ is the emission intensity when excited at the maximum excitation wavelength for the acceptor.

As such, the fluorescence emission intensity measured at $\lambda_{Ex} = 347$ nm was normalised to the emission intensity measured at $\lambda_{Ex} = 405$ nm. For **P3-Pyr₁₀₀ + P4-NI₂₀₀**, the calculated % FRET

was 56% , suggesting that the donor and acceptor dyes have been brought closer together, increasing the efficiency of photon transfer between the FRET pair. For this to occur, the membranes of the **P3-Pyr₁₀₀** and **P4-NI₂₀₀** particles must undergo a fusion process when particles collide, suggesting a hetero-fusion process is taking place.

The % FRET calculated for **P3-Pyr₁₀₀** + **P4-NI₁₀₀**, where the particles remain predominantly in an unfused, spherical morphology, was 35%. As these particles do not fuse, the donor and acceptor dyes are not brought into close proximity, and so the fluorescence emission at $\lambda_{\text{EX}} = 347 \text{ nm}$ cannot be due to the energy transfer between the FRET pair. It is known that pyrene forms dimers in the excited state, which result in a red-shifted emission band.⁴⁶ This shift may result in the emission band of the pyrene overlapping with the acceptor emission band, leading to a false positive when measuring the FRET response. This is a key limitation of this FRET pair and could be overcome by using other combinations reported in the literature (e.g. Cy3 and Cy5) in future work.⁴⁷

3.4 Conclusion

In this chapter, both negatively and positively charged polymersomes were synthesised with varying core DPs using ROMPISA. All samples were shown to have a spherical morphology by dry-state TEM, except for **P2₃₀₀**, for which a small degree of anisotropy was observed. This was attributed to increased membrane tension to a level that overcame the spontaneous fusion threshold.

Following this, oppositely charged polymersomes were mixed in a 1:1 ratio, varying the core DP of each constituent particle. An increase in average particle size was observed by DLS analysis, when compared to the original polymersome solutions. In addition, a range of morphologies were observed by dry-state TEM analysis, including linear tubes, branched structures, and irregular clusters. A significant size disparity in the original polymersomes led to the formation of cluster-like structures. This was attributed to the high energy barrier for smaller polymersomes to undergo linear fusion with larger polymersomes.

Furthermore, polymersomes of equivalent core DP were shown to fuse to the most significant degree, suggesting a possible size selectivity in charge-mediated polymersome fusion. During the chain rearrangement, polymers of equivalent core DP can pack in an arrangement that aligns the oppositely charged functional groups more closely, thus producing a structure lower in energy. In contrast, when core DP is not equivalent, the packing is less optimal, resulting in a higher energy product and less of a thermodynamic driving force for fusion. Fusion was observed when polymersomes below the critical core DP to fuse (**P1₁₀₀** and **P2₁₀₀**) were mixed, indicating electrostatic charge attraction can be utilised as an energetic driving force for polymersome fusion.

A model system of two diblock polymersomes synthesised by ROMPISA was used to investigate the mode of fusion upon mixing. Each type of polymersome was tagged with fluorescent dyes that formed a FRET pair when brought into close proximity. The fluorophores were incorporated into the polymersomes without impact on particle size, morphology or resulting fusion behaviour.

As such, fluorescence analysis following the mixing of the dye-containing polymersomes showed a greater FRET response when the particles were seen to fuse compared to an unfused system. This increase is caused by the two dyes being brought close to one another and therefore must be due to a hetero-fusion process, where fusion occurs between the two different particles.

The results shown here demonstrate the ability to utilise electrostatic attraction as a driving force for polymersome fusion, thus broadening the scope for synthetic systems to mimic the fusion behaviour observed in biology. In addition, determining the mode of fusion will provide further insights into the mechanism of polymersome fusion and drive future development for more complex systems.

3.5 Experimental

3.5.1 Materials

Unless otherwise stated reagents were obtained from commercial sources and used without purification. Tetrahydrofuran (THF) (HPLC grade) was purchased from VWR Chemicals and was purified *via* passage through a column of neutral alumina prior to use. Formvar-carbon coated (300 mesh) copper grids were purchased from EM Resolutions.

G3 (modified Grubbs 3rd generation catalyst, $((\text{H}_2\text{IMes})(\text{pyr})_2(\text{Cl})_2\text{Ru}=\text{CHPh}))$), **NB-PEG**, **NB-Amine**, **NB-MEG**, and exo-5-norbornene imide were prepared as previously described.^{34, 43, 48} **NB-COOH** was synthesised following the procedure outlined in Chapter 2, Section 2.5.3.1.

3.5.2 Characterisation Techniques

3.5.2.1 NMR Spectroscopy

^1H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker DPX-400 spectrometer in CDCl_3 or $(\text{CD}_3)_2\text{SO}$. Chemical shifts of protons are reported as δ in parts per million (ppm) and are relative to solvent residual peaks (CDCl_3 δ = 7.26 ppm, $(\text{CD}_3)_2\text{SO}$ δ = 2.50 ppm). All ^1H resonances are reported to the nearest 0.01 ppm. The multiplicity of ^1H signals are indicated as: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; or combinations of thereof. Coupling constants (J) are quoted in Hz and reported to the nearest 0.1 Hz. Where appropriate, averages of the signals from peaks displaying multiplicity were used to calculate the coupling constant value. ^{13}C NMR spectra were recorded on the same spectrometer with the central resonance of the solvent peak as the internal reference (CDCl_3 δ = 77.16 ppm). All ^{13}C resonances are reported to the nearest 0.01 ppm.

3.5.2.2 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) analysis was performed on a system composed of an Agilent 1260 Infinity II LC system equipped with an Agilent guard column (PLGel 5 μ M, 50 $\times$ 7.5 mm) and two Agilent Mixed-C columns (PLGel 5 μ M, 300 $\times$ 7.5 mm). The mobile phase used was THF (HPLC grade) containing 2% v/v NEt_3 at 40 $^\circ\text{C}$ with a flow rate of 1.0 mL min^{-1} (poly(methyl methacrylate) (PMMA) standards used for calibration). Number average molecular weight (M_n), weight average molecular weight (M_w) and dispersity ($\mathcal{D} = M_w/M_n$) were determined using Agilent GPC/SEC software.

3.5.2.3 Dynamic Light Scattering

Hydrodynamic diameters (D_h) and size distributions (PD) of nano-objects were determined by dynamic light scattering (DLS) and microelectrophoresis using a Malvern Zetasizer Nano ZS with a 4 mW He-Ne 633 nm laser module operating at 25 $^\circ\text{C}$. Samples were diluted to give a final polymer concentration of 0.01 wt%. Measurements were carried out at an angle of 173 $^\circ$ (back scattering), and results were analysed using Malvern DTS v7.03 software. All determinations were repeated five times, with at least 10 measurements recorded for each run. D_h values were calculated using the Stokes-Einstein equation, where particles are assumed to be spherical. In contrast, DLS was used for anisotropic particles to indicate a change in average particle size and obtain dispersity information.

3.5.2.4 Transmission Electron Microscopy

Dry-state, stained transmission electron microscopy (TEM) imaging was performed on a JEOL JEM-1400 microscope operating at an acceleration voltage of 80 kV. All dry-state samples were diluted to an appropriate analysis concentration and then deposited onto formvar-

coated grids. After roughly 1 min, the excess sample was blotted from the grid, and the grid was then stained with an aqueous 1 wt% uranyl acetate (UA) solution for 1 min before blotting, drying and microscopic analysis. Images were analysed using ImageJ software in which a minimum of 100 particles across a minimum of 3 images were measured.

3.5.2.5 UV-Vis Spectroscopy

UV-vis spectroscopy was performed on a Perkin Elmer Lambda 35 UV/vis spectrometer. Quartz cells (170-2000 nm) from Hellma with two polished sides were used for examining the absorption spectral data by using Thermo INSIGHT software.

3.5.2.6 Fluorescence Spectroscopy

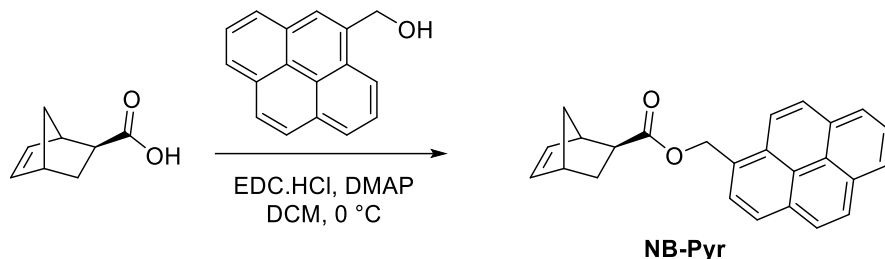
Fluorescence emission and excitation spectra were obtained using an Edinburgh Instruments FS5 Spectrofluorometer in quartz 3.5 mL cuvettes for liquid samples (Starna Cell, Type: 3/Q/10), and analysed in Fluoracle (Edinburgh Instruments).

3.5.2.7 High-Resolution Mass Spectrometry

High-Resolution Mass Spectrometry (HRMS) spectra were recorded by the MS Analytical Facility Service at the University of Birmingham on a Waters Xevo G2-XS QToF Quadrupole Time-of-Flight mass spectrometer.

3.5.3 Monomer Synthesis

3.5.3.1 Synthesis of *exo*-Norbornene pyrene (NB-Pyr)



Scheme S3.1. Synthesis of **NB-Pyr**.

Exo-5-norbornene-2-carboxylic acid (2 g, 14.5 mmol, 1 eq), 1-pyrenemethanol (4.04 g, 17.4 mmol, 1.2 eq), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) (3.33 g, 17.4 mmol, 1.2 eq) and 4-dimethylaminopyridine (DMAP) (0.88 g, 7.3 mmol, 0.5 eq) were added to a flask and cooled to 0 °C. CH₂Cl₂ (60 mL) was added to the flask, stirring the solution for 30 mins at 0 °C. After warming to room temperature, the solution was stirred for a further 18 hours. The crude product was dissolved in CH₂Cl₂ and washed with H₂O (x3) and brine. The organic layer was dried over MgSO₄, filtered, and concentrated to dryness under reduced pressure. The crude product was purified further *via* column chromatography using silica gel as the stationary phase and a mixture of 10% ethyl acetate in petroleum ether as the eluent to afford the pure product **NB-Pyr** as a fluorescent pale-yellow solid (3.32 g, 65%). ¹H NMR (400 MHz, CDCl₃) δ 8.31 – 7.98 (m, 9H), 6.10 (m, 1H), 6.05 (ddd, *J* = 5.8, 3.1, 0.7 Hz, 1H), 5.87 (m, 2H), 3.08 (dtt, *J* = 2.9, 1.4, 0.8 Hz, 1H), 2.91 (ddq, *J* = 2.9, 1.9, 1.0 Hz, 1H), 2.31 (m, 1H), 1.98 (m, 1H), 1.56 (dtd, *J* = 7.7, 1.6, 1.0 Hz, 1H), 1.42 – 1.32 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 207.05, 176.33, 138.18, 135.84, 131.82, 131.33, 130.83, 129.64, 129.31, 128.27, 127.90, 127.85, 127.49, 126.20, 125.62, 125.54, 125.02, 124.79, 124.75, 123.08, 64.95, 46.81, 46.53, 43.41, 41.79, 31.05, 30.53. MS: *m/z* [M]⁺ calc. 352.15 g mol⁻¹, exp. 352.14 g mol⁻¹.

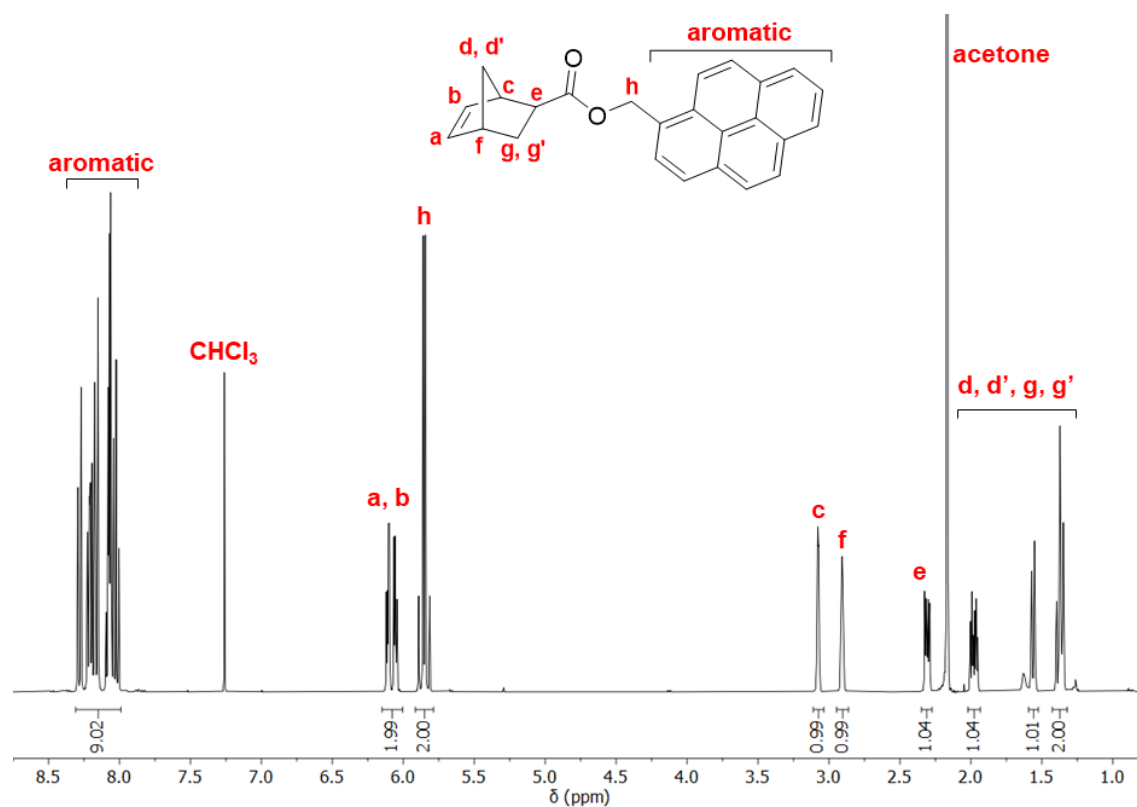


Figure S3.1. ¹H NMR spectrum (400 MHz, CDCl₃, 298 K) of NB-Pyr.

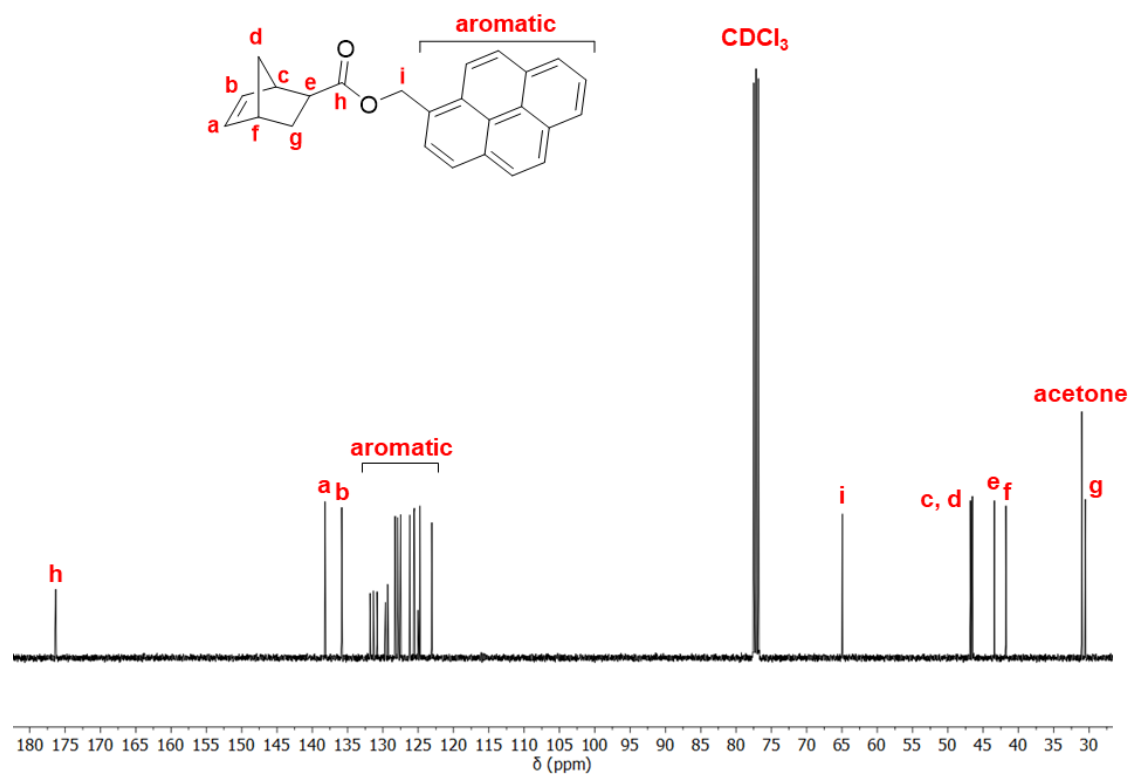
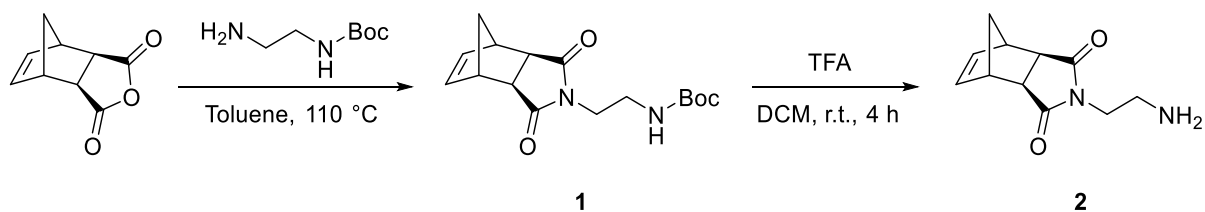
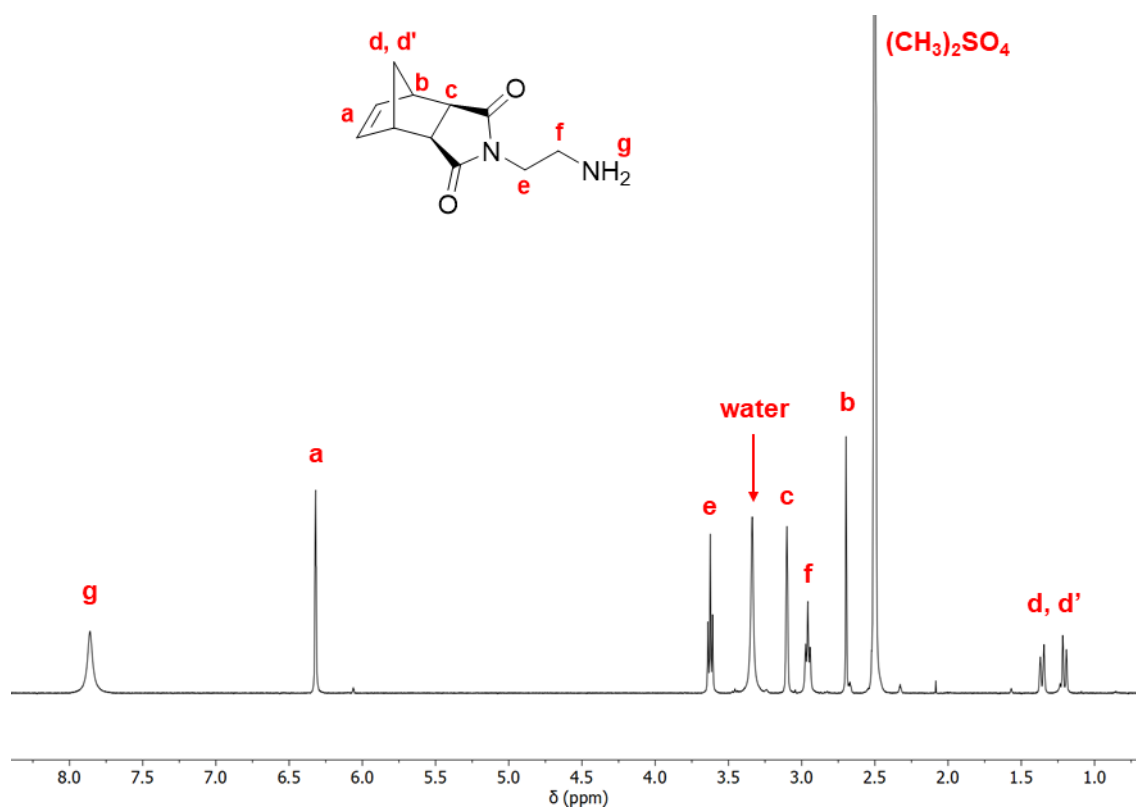
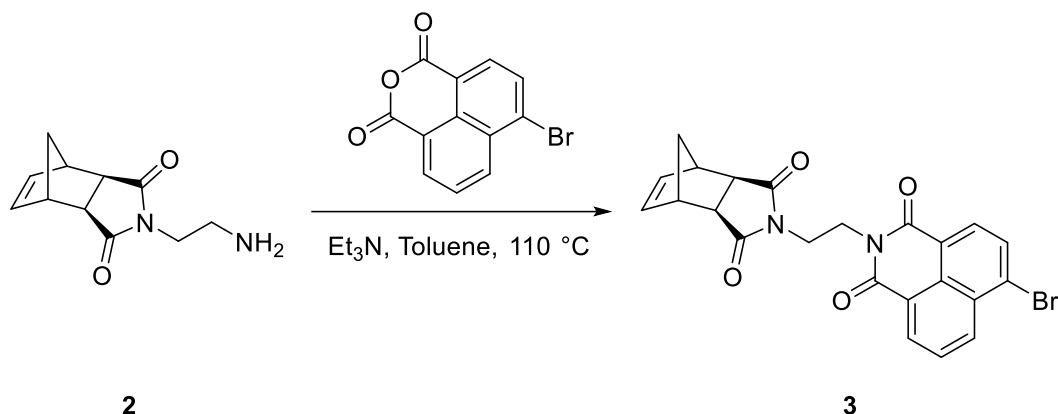


Figure S3.2. ¹³C NMR spectrum (101 MHz, CDCl₃, 298 K) of NB-Pyr.

3.5.3.2 Synthesis of *exo*-Norbornene imide morpholine naphthalimide (NB-NI)**Scheme S3.2.** Synthesis of intermediate **2**.

The synthesis of the amine-functionalised norbornene imide precursor **2** was carried out according to a previously described process.⁴⁹ ^1H NMR (400 MHz, $(\text{CH}_3)_2\text{SO}_4\text{-}d_6$) δ 7.86 (s, 3H), 6.32 (t, $J = 1.9$ Hz, 2H), 3.62 (t, $J = 6.4$ Hz, 2H), 3.10 (t, $J = 1.8$ Hz, 2H), 2.96 (t, $J = 6.5$ Hz, 2H), 2.70 (d, $J = 1.3$ Hz, 2H), 1.36 (dt, $J = 9.8, 1.7$ Hz, 1H), 1.20 (d, $J = 9.7$ Hz, 1H).

**Figure S3.3.** ^1H NMR spectrum (400 MHz, $(\text{CH}_3)_2\text{SO}_4\text{-}d_6$, 298 K) of intermediate **2**.



Scheme S3.3. Synthesis of intermediate **3** from **2**.

4-Bromo-1, 8-naphthalic anhydride (112 mg, 0.40 mmol, 1 eq), amine *exo*-norbornene imide **2** (100 mg, 0.48 mmol, 1.2 eq) were dissolved in toluene (15 mL). Et₃N (123 mg, 1.21 mmol, 3 eq) was added, and the solution was refluxed for 2 h. Upon cooling to room temperature, the reaction mixture was concentrated to dryness under reduced pressure. The crude product was then purified *via* column chromatography using silica gel as the stationary phase and a mixture of hexane/ethyl acetate 3:1 as the eluent to afford the pure intermediate **3** as a white solid (157 mg, 84%). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.62 (dd, *J* = 7.3, 1.1 Hz, 1H), 8.58 (dd, *J* = 8.5, 1.2 Hz, 1H), 8.38 (d, *J* = 7.9 Hz, 1H), 8.03 (d, *J* = 7.9 Hz, 1H), 7.84 (dd, *J* = 8.5, 7.3 Hz, 1H), 6.21 (t, *J* = 1.9 Hz, 2H), 4.45 – 4.40 (m, 2H), 3.97 – 3.92 (m, 2H), 3.16 (p, *J* = 1.7 Hz, 2H), 2.60 (d, *J* = 1.4 Hz, 2H), 1.47 (dt, *J* = 9.9, 1.6 Hz, 1H), 1.29 (dtt, *J* = 9.8, 1.4, 0.6 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 178.37, 163.83, 137.93, 133.68, 132.23, 131.40, 131.27, 130.52, 130.73, 130.39, 129.65, 128.24, 123.10, 122.10, 48.09, 45.07, 43.10, 38.94, 37.28.

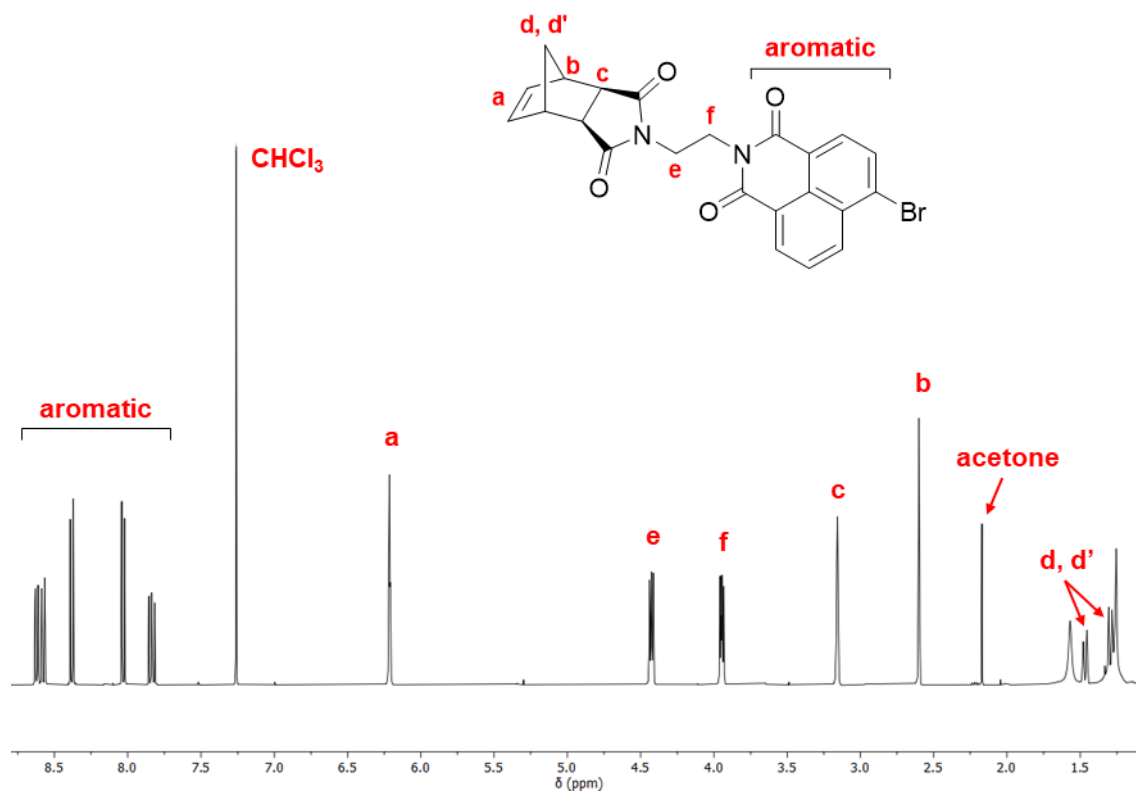


Figure S3.4. ^1H NMR spectrum (400 MHz, CDCl_3 , 298 K) of intermediate **3**.

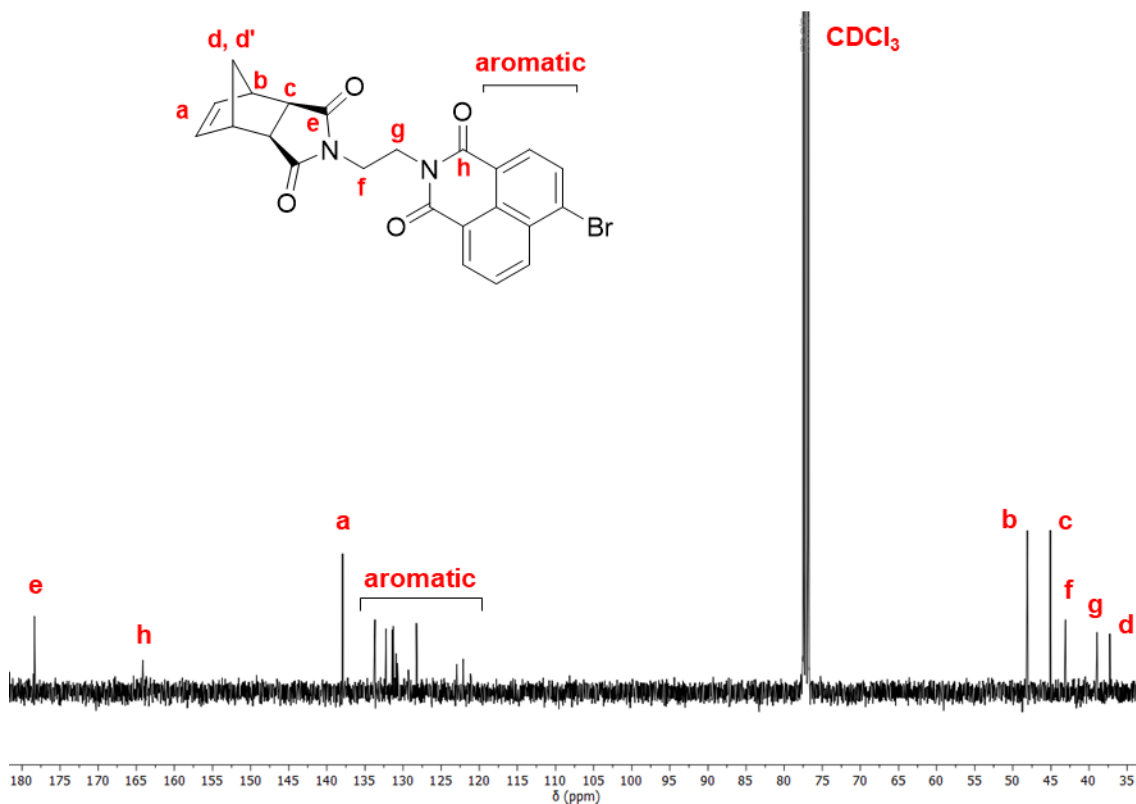
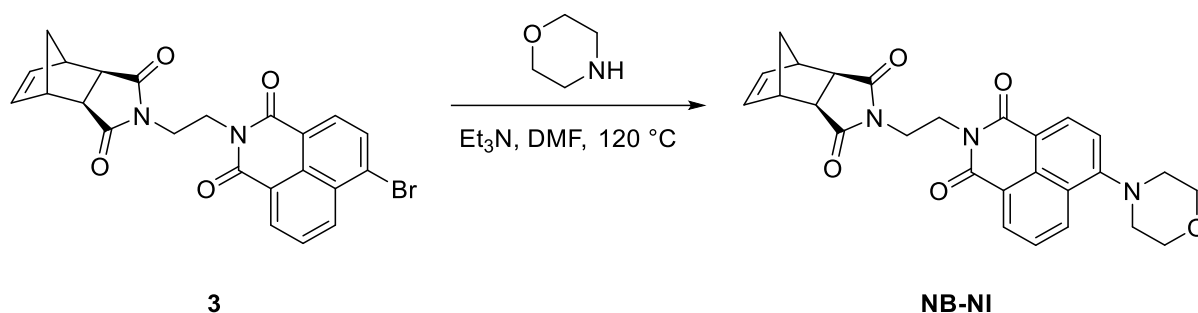


Figure S3.5. ^{13}C NMR spectrum (101 MHz, CDCl_3 , 298 K) of intermediate **3**.



Scheme S3.4. Synthesis of **NB-NI** from intermediate **3**.

3 (100 mg, 0.21 mmol, 1 eq) was dissolved in DMF (15 mL). Morpholine (37.4 mg, 0.43 mmol, 2 eq) and Et_3N (54.4, 0.54 mmol, 2.5 eq) were added and the solution was refluxed for 3 h. Upon cooling to room temperature, the reaction mixture was concentrated to dryness under reduced pressure. The crude product was then purified *via* column chromatography using silica gel as the stationary phase and a mixture of 10-30% ethyl acetate in CH_2Cl_2 as the eluent to afford the pure product **NB-NI** as a white solid (63.9 mg, 65%). ^1H NMR (400 MHz, Chloroform-*d*) δ 8.55 (dd, $J = 7.2, 1.2$ Hz, 1H), 8.49 (d, $J = 8.0$ Hz, 1H), 8.41 (dd, $J = 8.5, 1.2$ Hz, 1H), 7.68 (dd, $J = 8.4, 7.3$ Hz, 1H), 7.21 (d, $J = 8.1$ Hz, 1H), 6.21 (t, $J = 1.9$ Hz, 2H), 4.45 – 4.39 (m, 2H), 4.05 – 3.99 (m, 4H), 3.97 – 3.91 (m, 2H), 3.29 – 3.23 (m, 4H), 3.16 (p, $J = 1.7$ Hz, 2H), 2.61 (d, $J = 1.4$ Hz, 2H), 1.47 (dt, $J = 9.9, 1.6$ Hz, 1H), 1.33 – 1.29 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 178.26, 164.77, 164.27, 155.81, 137.79, 132.57, 131.22, 130.27, 126.30, 125.83, 123.05, 116.88, 115.00, 67.00, 53.46, 47.97, 44.91, 43.00, 38.51, 37.29. MS: m/z $[\text{M}+\text{Na}]^+$ calc. 494.17 g mol^{-1} , exp. 494.17 g mol^{-1} .

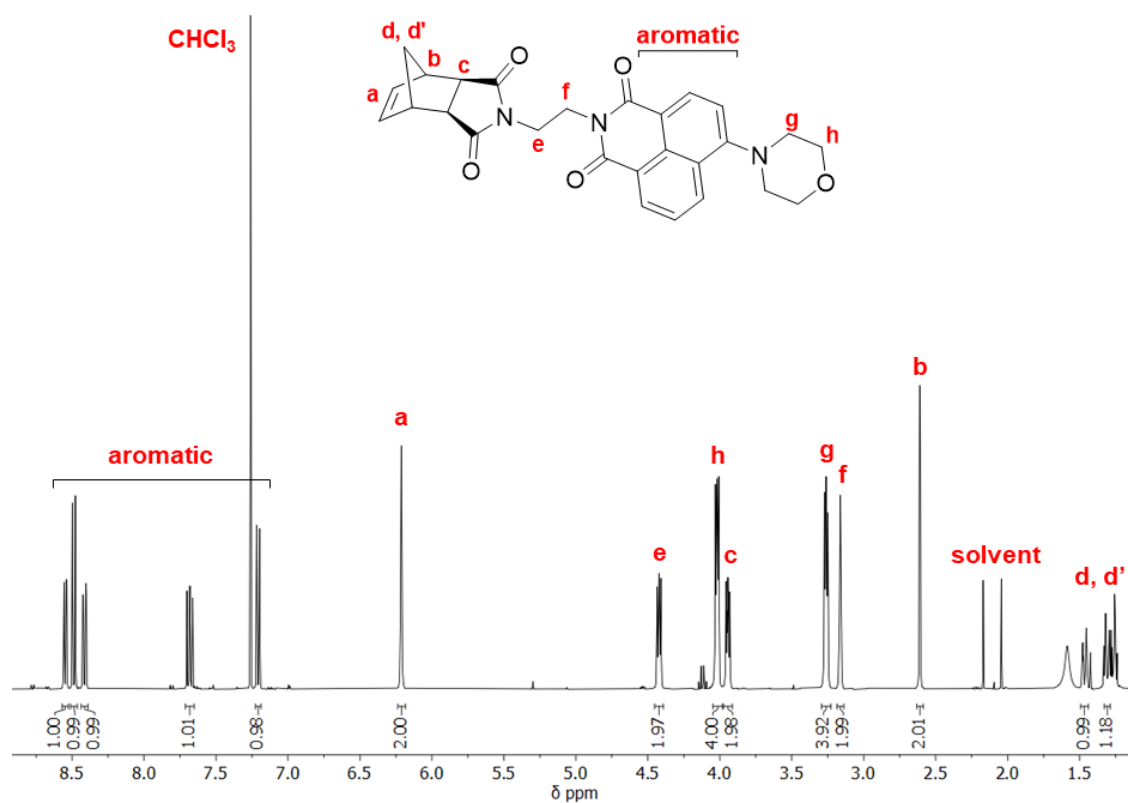


Figure S3.6. ¹H NMR spectrum (400 MHz, CDCl₃, 298 K) of NB-NI.

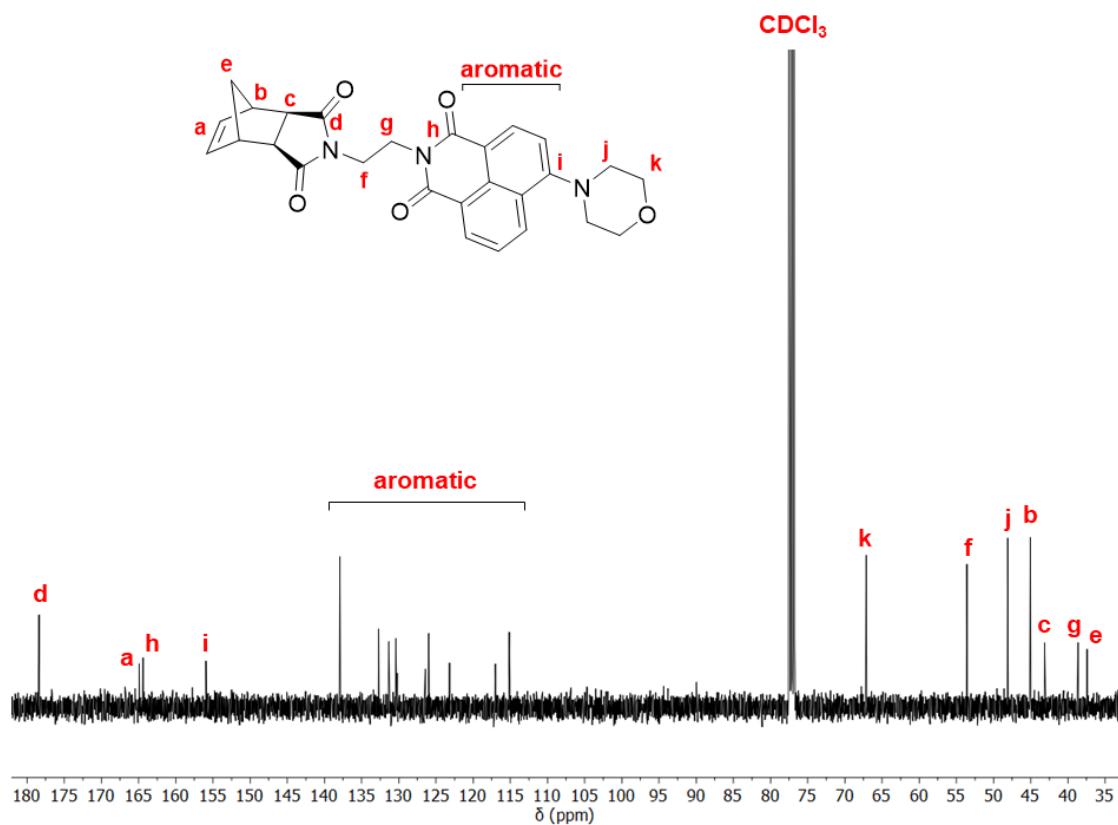


Figure S3.7. ¹³C NMR spectrum (400 MHz, CDCl₃, 298 K) of NB-NI.

3.5.4 Mixing oppositely charged nano-objects synthesised *via* ROMPISA

3.5.4.1 Synthesis of $P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-Amine})_5\text{-}b\text{-}P(\text{NB-MEG})_y$ in salt solution, $P2_y$

$P2_y$ nano-objects were synthesised analogous to reference 34.

Table S3.1. SEC data for intermediate polymers for $P2_y$.

Polymer	$M_{n,\text{theo}}$ / kDa	$M_{n,\text{GPC}}$ / kDa	$\bar{D}_{\text{GPC}}$
$P(\text{NB-PEG})_{11}$	6.6	6.8	1.13
$P(\text{NB-PEG})_{11}\text{-}b\text{-}P(\text{NB-Amine})_5$	7.7	8.2	1.12

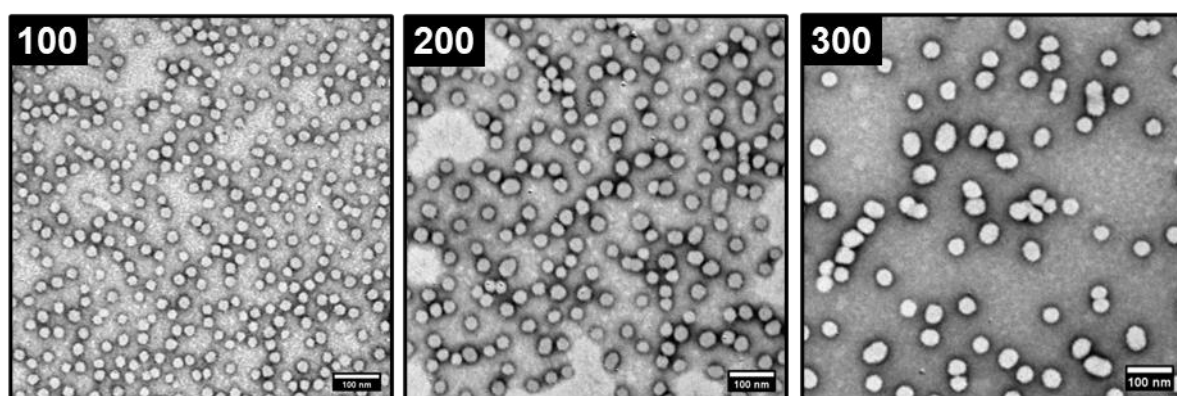


Figure S3.8. Supplementary dry-state TEM images of $P2_y$ particles: $y = 100, 200,$ and 300 . Scale bar = 100 nm.

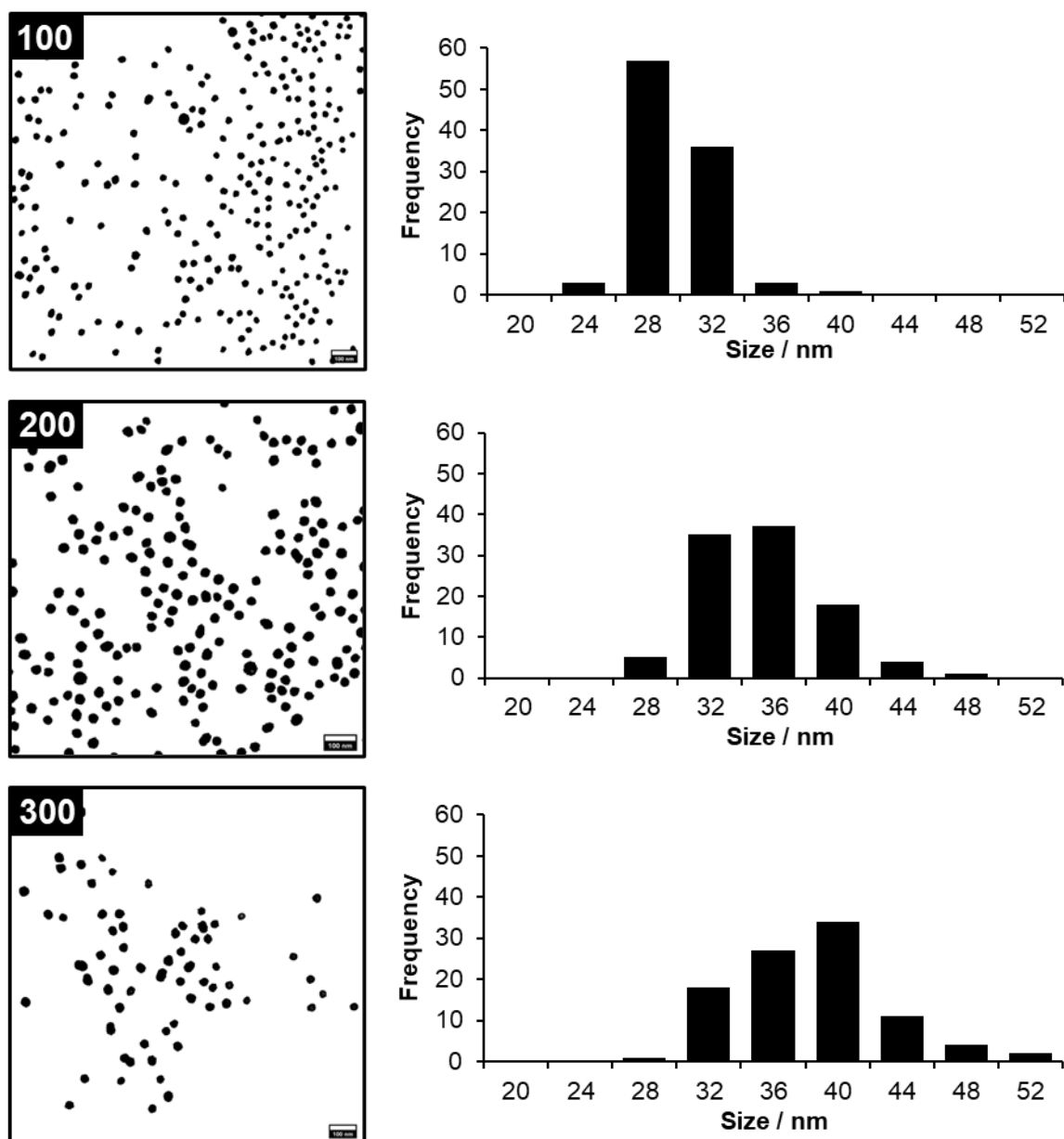


Figure S3.9. Dry-state TEM image analysis results for $P2_y$ particles synthesised at pH 7: $y = 100, 200,$ and 300 , as determined by image processing using ImageJ analysis software.

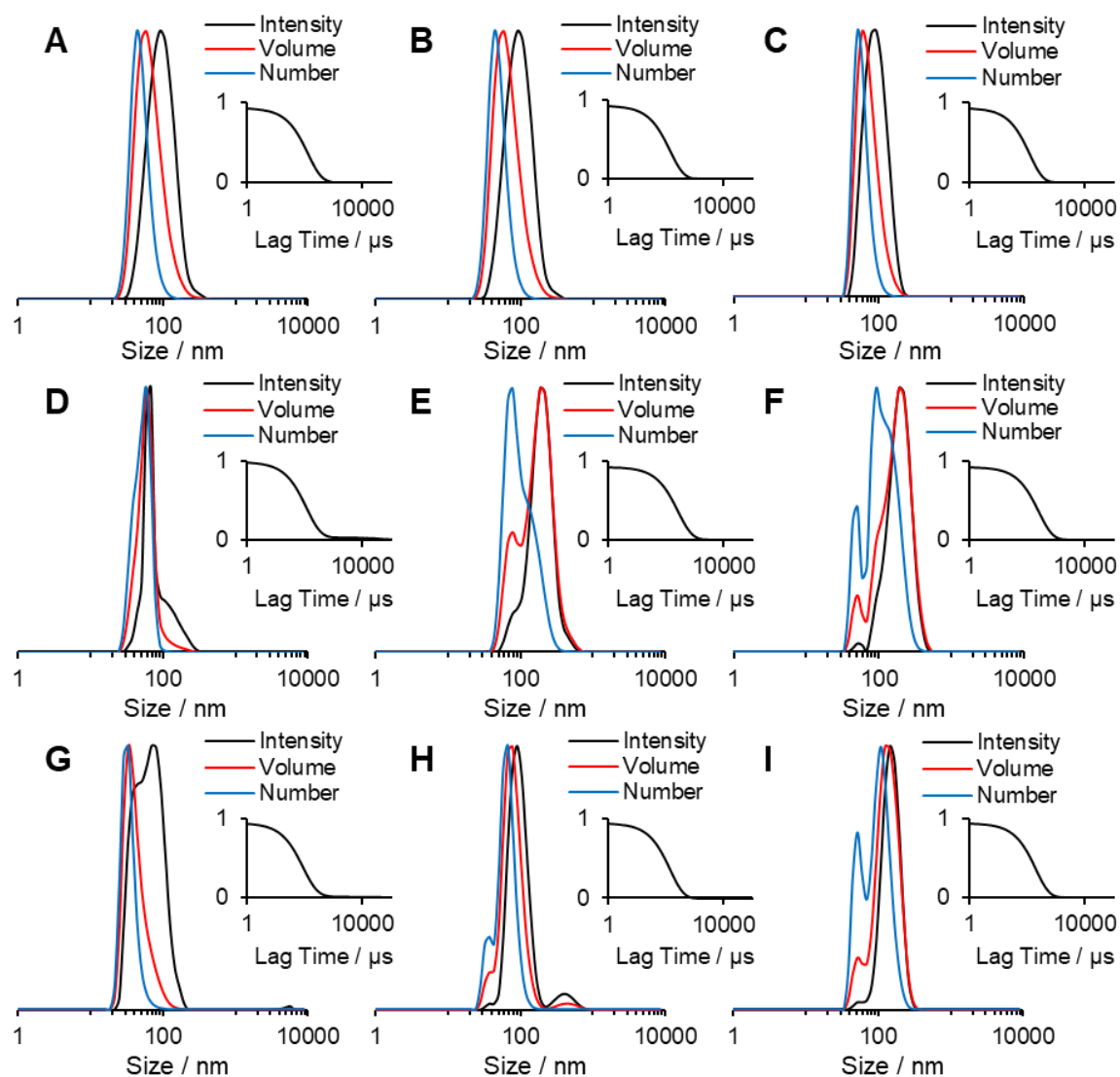
3.5.4.2 Mixing P1_x and P2_y nano-objects

Figure S3.10. Normalised DLS analysis of (A) $P1_{100} + P2_{100}$, (B) $P1_{100} + P2_{200}$, (C) $P1_{100} + P2_{300}$, (D) $P1_{200} + P2_{100}$, (E) $P1_{200} + P2_{200}$, (F) $P1_{200} + P2_{300}$, (G) $P1_{300} + P2_{100}$, (H) $P1_{300} + P2_{200}$ and (I) $P1_{300} + P2_{300}$.

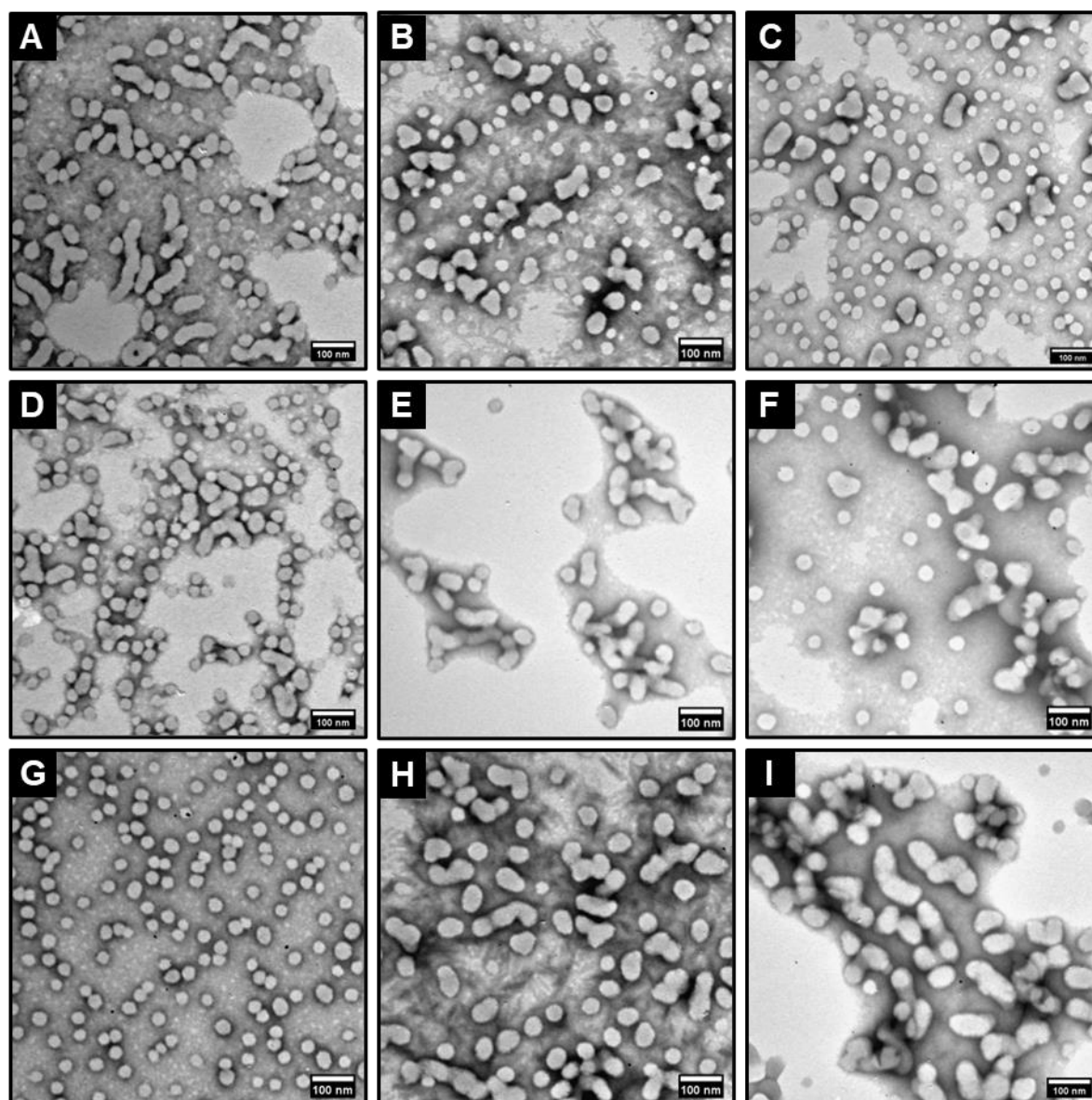


Figure S3.11. Supplementary dry-state TEM images of (A) $P1_{100} + P2_{100}$, (B) $P1_{100} + P2_{200}$, (C) $P1_{100} + P2_{300}$, (D) $P1_{200} + P2_{100}$, (E) $P1_{200} + P2_{200}$, (F) $P1_{200} + P2_{300}$, (G) $P1_{300} + P2_{100}$, (H) $P1_{300} + P2_{200}$ and (I) $P1_{300} + P2_{300}$.

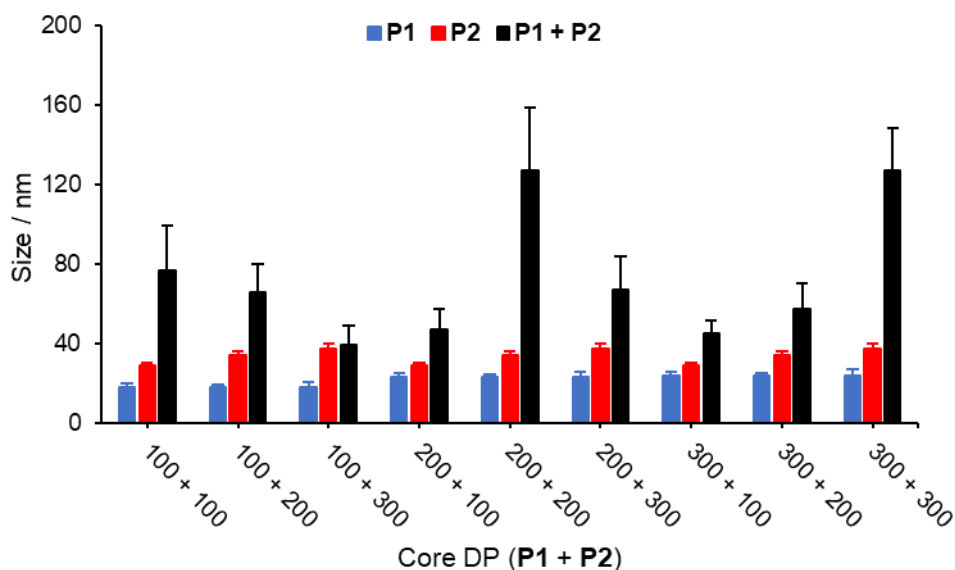


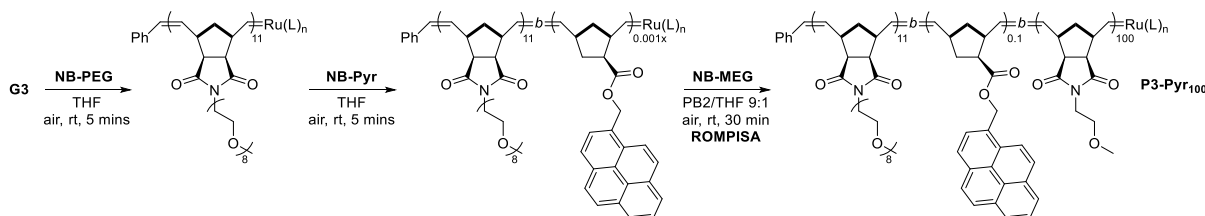
Figure S3.12. Average particle size of **P1_x** (blue), **P2_y** (red) and **P1_x + P2_y** (black), as determined by dry-state TEM analysis.

3.5.5 Mode of fusion determination

3.5.5.1 Synthesis of nano-objects *via* ROMPISA

P3₁₀₀ and **P4₂₀₀** nano-objects were synthesised according to reference 42.

Fluorescently labelled **P3** and **P4** nano-objects were synthesised analogous to the triblock nano-object preparation described in Chapter 2, Section 2.5.4.1. An example procedure for **P3-Pyr₁₀₀** is described below.

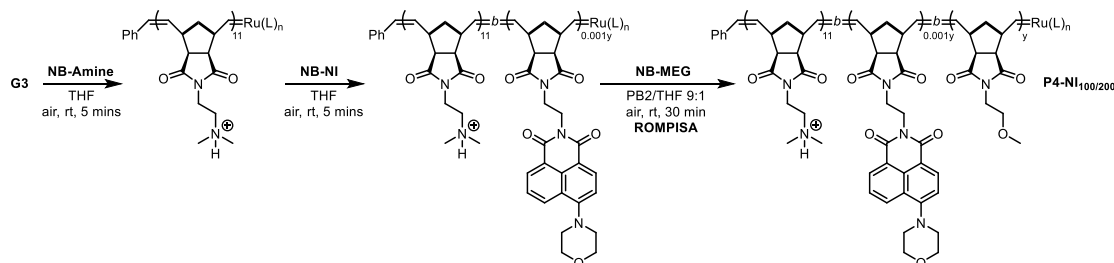


Scheme S3.5. Synthetic route for the preparation of **P3-Pyr₁₀₀** block copolymers by ROMPISA.

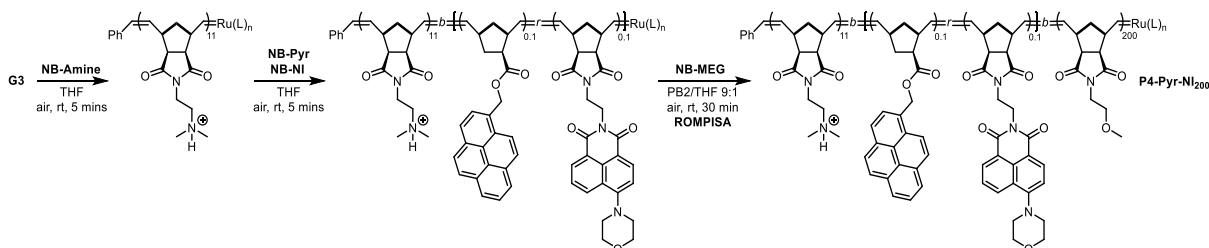
A solution of **NB-PEG** (31.2 mg, 59 μ mol, 13 eq.) in 530 μ L of filtered THF was rapidly added to a solution of **G3** (3.9 mg, 5.2 μ mol, 1.2 eq.) in 120 μ L of THF contained within a glass vial

equipped with a stirrer bar. The resulting solution was stirred rapidly for five minutes. After this time, a 100 μL aliquot was removed for GPC analysis, leaving 1.0 eq. of $\text{P}(\text{NB-PEG})_{11}$ in 550 μL of THF. To this solution was added **NB-Pyr** (0.16 mg, 0.46 μmol , 0.1 eq.) in 450 μL THF. The resulting solution was stirred for a further five minutes to give $\text{P}(\text{NB-PEG})_{11}\text{-}b\text{-P}(\text{NB-Pyr})_{0.1}$ macroinitiator (final concentration = 4.4 $\mu\text{mol/mL}$).

100 μL (0.44 μmol) of the resulting solution of $\text{P}(\text{NB-PEG})_{11}\text{-}b\text{-P}(\text{NB-Pyr})_{0.1}$ in THF was dispensed into a 2 mL glass vial containing a stirrer bar. A solution of **NB-MEG** (10 mg, 45 μmol , 100 eq.) in 900 μL of acidic phosphate buffer (pH = 2, PB2, final solids concentration = 1 wt%) was added rapidly to the vial. The resulting solution was thoroughly mixed by drawing up the entire volume into the pipette tip and ejecting the liquid back into the vial three times. The ROMPISA polymerisation was stirred at 420 rpm for 30 minutes to give $\text{P}(\text{NB-PEG})_{11}\text{-}b\text{-P}(\text{NB-Pyr})_{0.1}\text{-}b\text{-P}(\text{NB-MEG})_{100}$ (**P3-Pyr₁₀₀**). This was analysed by SEC, DLS and TEM.



Scheme S3.6. Synthetic route for the preparation of **P4-NI_{100/200}** block copolymers by ROMPISA.



Scheme S3.7. Synthetic route for the preparation of **P4-Pyr-NI₂₀₀** block copolymers by ROMPISA.

Table S3.2. SEC data of intermediate polymers for **P3** and **P4**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\bar{D}_{GPC}$
P(NB-PEG)₁₁	6.6	6.6	1.12
P(NB-Amine)₁₁	3.4	3.5	1.13
P(NB-PEG)₁₁-<i>b</i>-P(NB-Pyr)_{0.1}	6.6	6.6	1.12
P(NB-Amine)₁₁-<i>b</i>-P(NB-NI)_{0.1}	3.4	3.5	1.13
P(NB-Amine)₁₁-<i>b</i>-P(NB-NI)_{0.2}	3.5	3.6	1.13
P(NB-Amine)₁₁-<i>b</i>-[P(NB-Pyr)_{0.1}-<i>r</i>-P(NB-NI)_{0.1}]	3.4	3.6	1.13

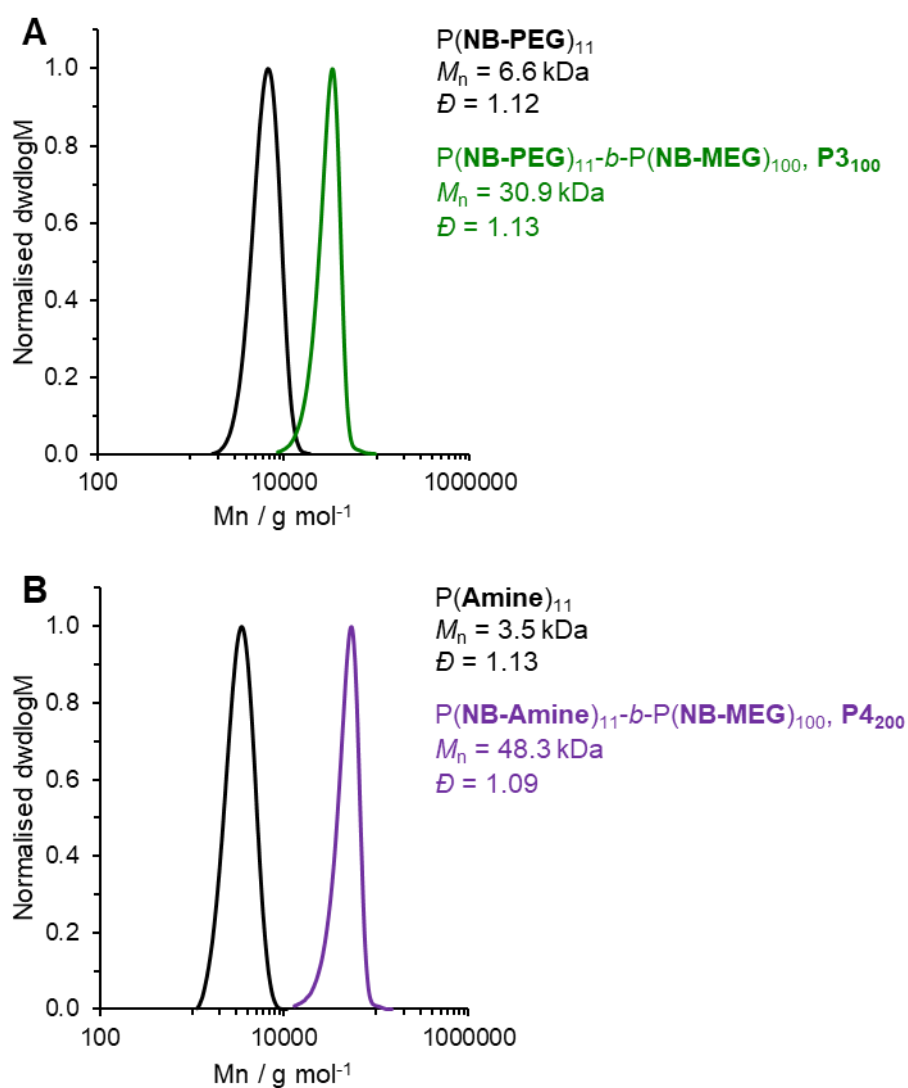


Figure S3.13. Normalised SEC traces (THF eluent, PMMA standards) of (A) **P3₁₀₀** and (B) **P4₂₀₀** and intermediate polymers.

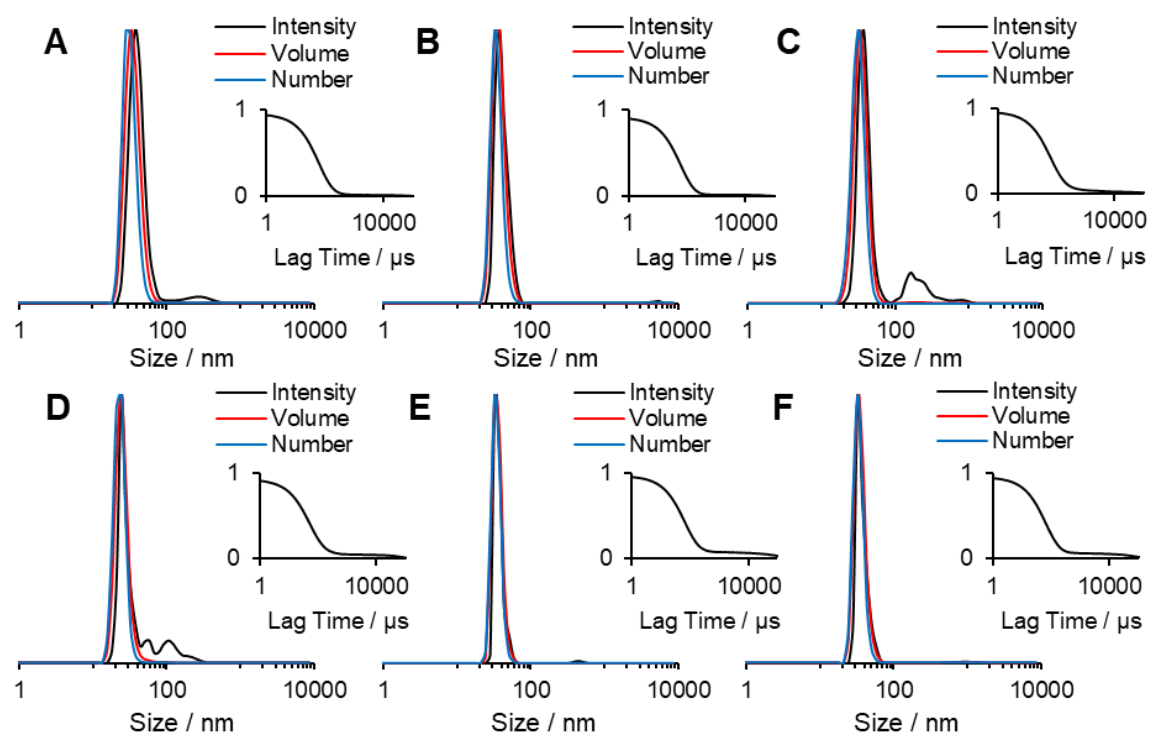


Figure S3.14. Normalised DLS analysis of (A) $P3_{100}$, (B) $P4_{200}$, (C) $P3-Pyr_{100}$, (D) $P4-NI_{100}$, (E) $P4-NI_{200}$ and (F) $P4-Pyr-NI_{200}$.

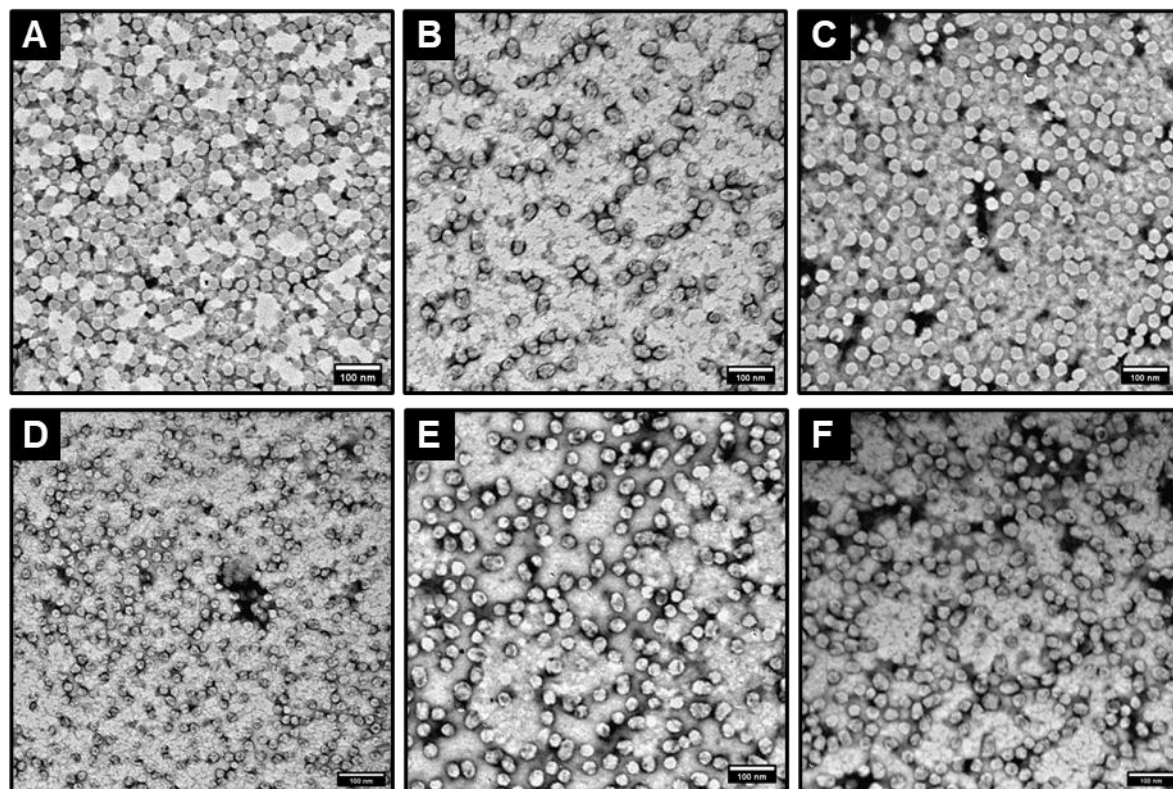


Figure S3.15. Supplementary dry-state TEM images of (A) **P3₁₀₀**, (B) **P4₂₀₀**, (C) **P3-Pyr₁₀₀**, (D) **P4-NI₁₀₀**, (E) **P4-NI₂₀₀**, (F) **P4-Pyr-NI₂₀₀**. Scale bar = 100 nm.

3.5.5.2 Mixing P3 and P4

P3 and **P4** nano-objects were mixed following the procedure previously described in Section 3.5.5

Table S3.3. Formulation details for **P3+ P4** mixing experiments.

Mixture	Particle 1	Particle 2
P3₁₀₀ + P4₂₀₀	P3₁₀₀	P4₂₀₀
P3₁₀₀ + P4-NI₂₀₀	P3₁₀₀	P4-NI₂₀₀
P3₁₀₀ + P4-Pyr-NI₂₀₀	P3₁₀₀	P4-Pyr-NI₂₀₀
P3-Pyr₁₀₀ + P4₂₀₀	P3-Pyr₁₀₀	P4₂₀₀
P3-Pyr₁₀₀ + P4-NI₁₀₀	P3-Pyr₁₀₀	P4-NI₁₀₀
P3-Pyr₁₀₀ + P4-NI₂₀₀	P3-Pyr₁₀₀	P4-NI₂₀₀

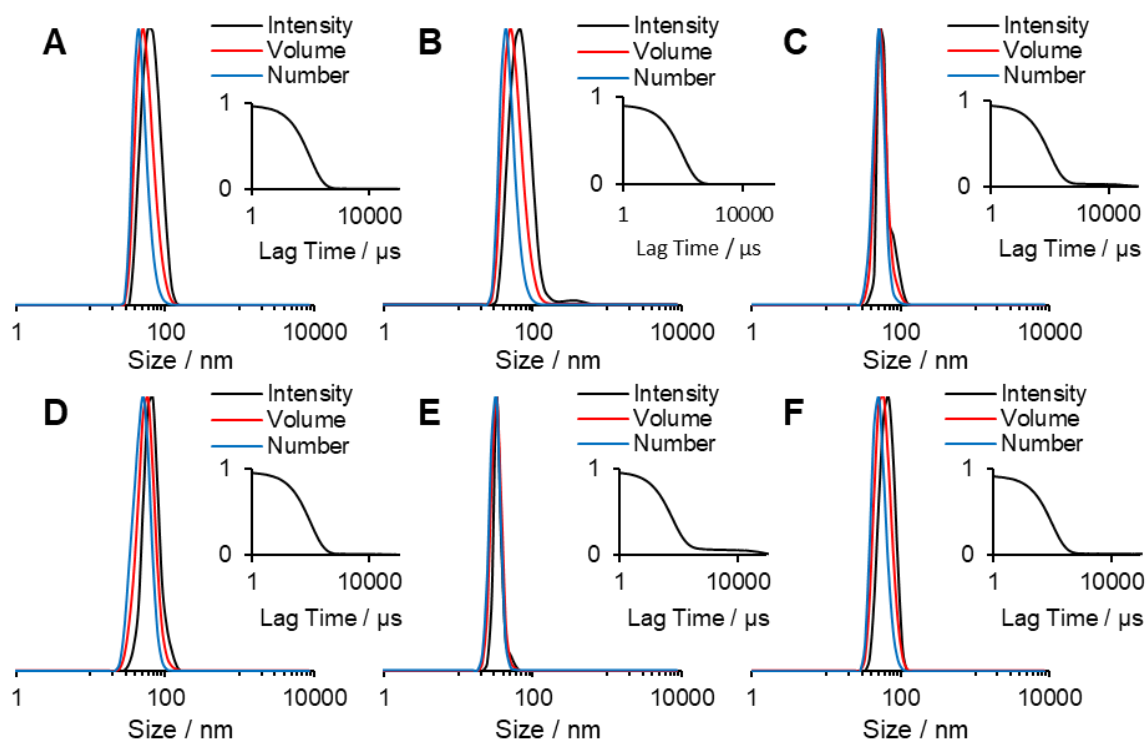


Figure S3.16. Normalised DLS analysis of (A) $\text{P3}_{100} + \text{P4}_{200}$, (B) $\text{P3}_{100} + \text{P4-NI}_{200}$, (C) $\text{P3}_{100} + \text{P4-Pyr-NI}_{200}$, (D) $\text{P3-Pyr}_{100} + \text{P4}_{200}$, (E) $\text{P3-Pyr}_{100} + \text{P4-NI}_{100}$ and (F) $\text{P3-Pyr}_{100} + \text{P4-NI}_{200}$.

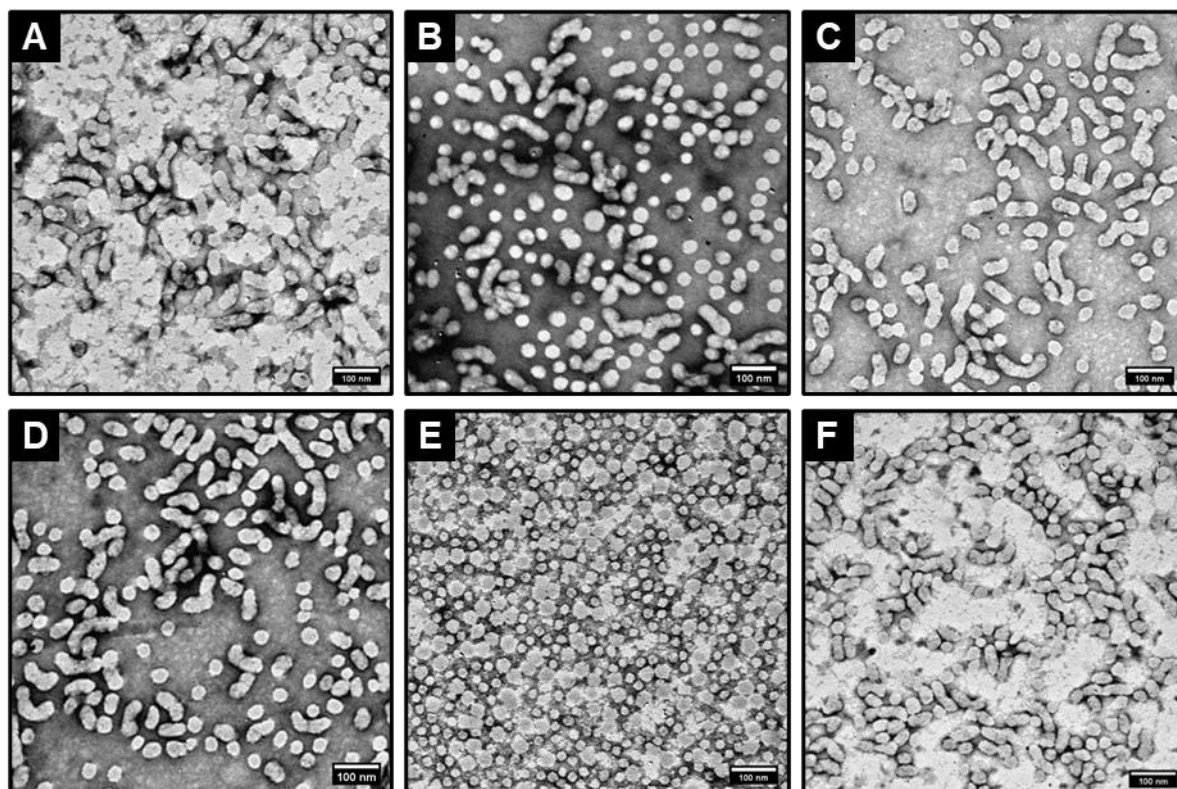


Figure S3.17. Supplementary dry-state TEM images of (A) $P3_{100} + P4_{200}$, (B) $P3_{100} + P4-NI_{200}$, (C) $P3_{100} + P4-Pyr-NI_{200}$, (D) $P3-Pyr_{100} + P4_{200}$, (E) $P3-Pyr_{100} + P4-NI_{100}$ and (F) $P3-Pyr_{100} + P4-NI_{200}$.

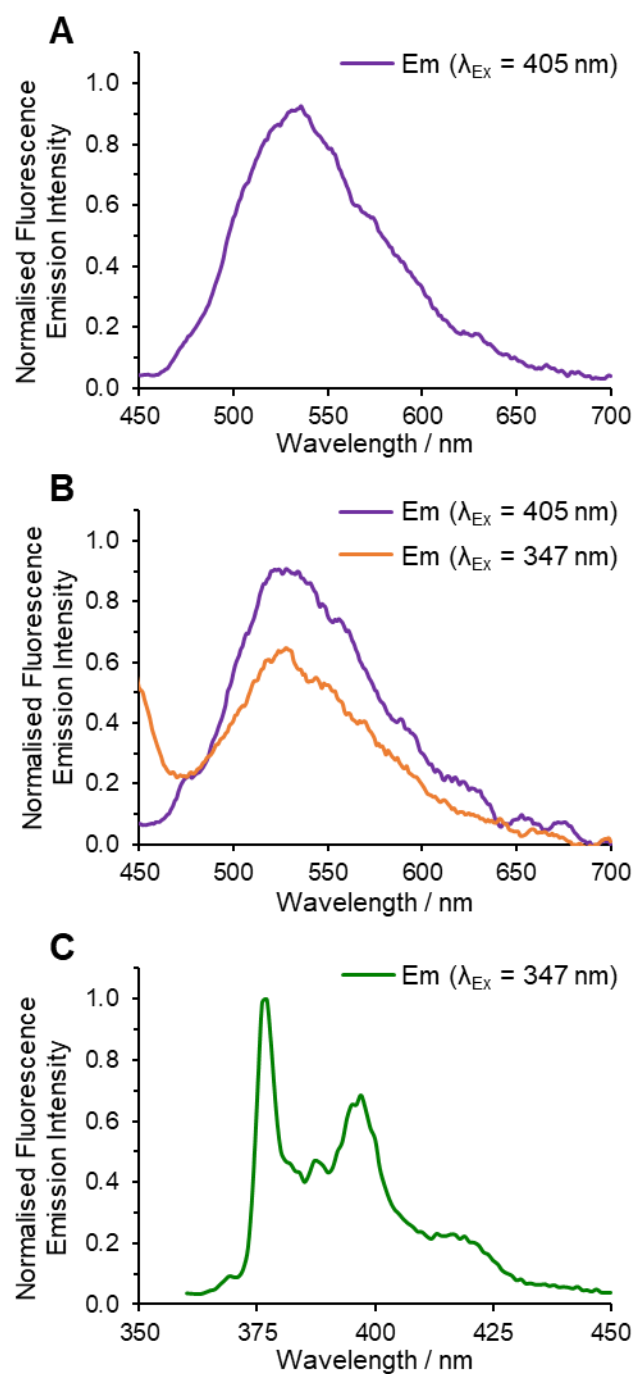


Figure S3.18. Normalised fluorescence emission spectra of (A) **P3**₁₀₀ + **P4-NI**₂₀₀, (B) **P3**₁₀₀ + **P4-Pyr-NI**₂₀₀ and (C) **P3-Pyr**₁₀₀ + **P4**₂₀₀. Green lines refer to **NB-Pyr** fluorescence ($\lambda_{\text{Ex}} = 347 \text{ nm}$), purple lines refer to **NB-NI** fluorescence ($\lambda_{\text{Ex}} = 405 \text{ nm}$), and orange lines refer to **NB-NI** fluorescence ($\lambda_{\text{Ex}} = 347 \text{ nm}$).

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**Chapter 4. Exploring the Encapsulation
of Cargo Within Nano-Objects
Synthesised *via* Aqueous Ring-Opening
Metathesis Polymerisation Induced
Self-Assembly**

4.1 Abstract

Encapsulation within nano-objects is a key property in determining their efficacy in a range of applications (e.g. drug-delivery vehicles). Encapsulation of water-soluble cargo has been demonstrated in nano-objects formed by different self-assembly techniques, most notably RAFT PISA. In contrast, the encapsulation efficiency of nano-objects synthesised *via* ring-opening metathesis polymerisation induced self-assembly (ROMPISA) is yet to be reported.

Herein, we explore the encapsulation of different water-soluble cargos within nano-objects prepared by ROMPISA. Concentration of cargo and nano-object composition are varied to determine the resulting effect on encapsulation efficiency. Analysis by bulk techniques and direct visualisation demonstrates that encapsulation is not observed during nano-object self-assembly.

The mechanism of formation of a model ROMPISA system is probed by time-resolved SAXS analysis. Spherical nano-objects are observed throughout the ROMPISA process, suggesting no anisotropic particles are formed. As such, these results indicate that ROMPISA proceeds *via* an alternative mechanism to RAFT PISA, thus resulting in highly inefficient encapsulation during nano-object formation.

4.2 Introduction

Compartmentalisation of material into discrete environments by semi-permeable structures is ubiquitous in biological systems. This endows these systems with numerous benefits, such as protection of sensitive material, faster rates of reaction, and the ability to perform incompatible processes simultaneously.¹ This is of key importance for processes that require harsh conditions.

For example, lysosomes are organelles that contain hydrolytic enzymes that are used for the controlled intracellular digestion of macromolecules, such as proteins, nucleic acids and carbohydrates.² The enzymes are acid hydrolases which are only active at acidic pH (about 4.5).³ The segregation of these degradative biomolecules provides the ideal environment for these enzymes whilst also protecting against the uncontrolled digestion of cell material.⁴

4.2.1 Polymersome encapsulation

Using this as inspiration, encapsulation of material (or cargo) within polymersomes has been widely reported.⁵ In particular, encapsulation of molecules with the ability to provide information on their environment has allowed for a greater understanding of block copolymer self-assembly. This method can be used to extract information about the polymersome itself (e.g. membrane permeability), as well as probe their effectiveness for different processes that would be desirable in potential applications (e.g. response to stimuli). As such, the encapsulation of fluorescent dyes within polymersomes has been highly reported.

For example, Xie and co-workers reported the use of an acid-sensitive fluorescent dye to quantify the permeability of protons through polymersome membranes.⁶ Poly(ethylene glycol)-*b*-poly(caprolactone) (PEG-*b*-PCL) block copolymers were first synthesised by ring-

opening polymerisation (ROP), followed by self-assembly in water *via* nanoprecipitation in the presence of boron-dipyrromethene (BODIPY). Being hydrophobic in nature, the BODIPY dye was situated within the membrane core (Figure 4.1).

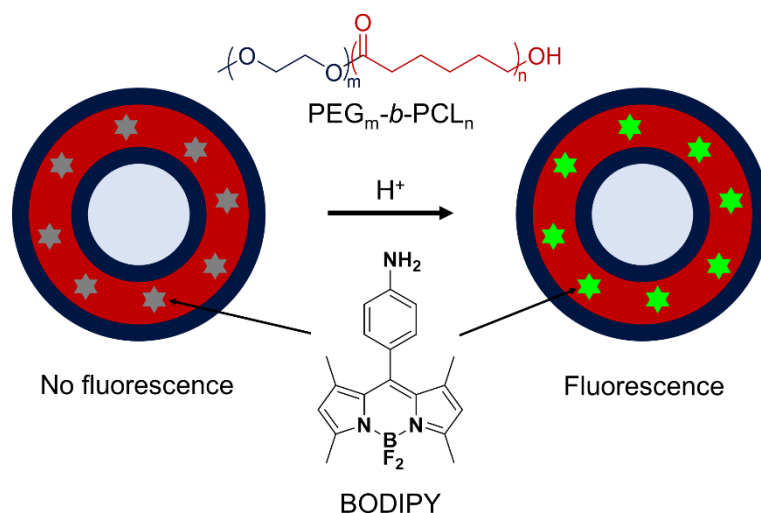


Figure 4.1. Schematic diagram of pH-sensitive BODIPY encapsulated within the membrane core of PEG-*b*-PCL polymersomes and its fluorescence switch on upon a decrease in pH. Adapted from Quan *et al.*⁶

Known amounts of trifluoroacetic acid (TFA) was added to the aqueous solution of polymersomes and the fluorescence emission intensity measured at regular time intervals. An exponential increase in emission intensity was observed over time indicating diffusion-controlled fluorescence enhancement. Further to this, they investigated the impact of additional PCL stabiliser during self-assembly on the resulting membrane permeability. The results showed a lower permeability for the polymersomes with a greater membrane thickness, following a linear trend, thus indicating the membrane permeability can be tuned for PEG-*b*-PCL polymersomes.

Adaptive polymersomes, often referred to as “smart polymersomes”, are able to change their properties in response to a stimulus.⁷ For example, Boesel and Bruns synthesised

pentafluorophenyl methacrylate-*b*-hexyl methacrylate (P(PFPMA)-*b*-P(HMA)) polymersomes, functionalised with donor–acceptor Stenhouse adducts (DASA) (Figure 4.2).⁸ These were used to encapsulate horseradish peroxidase (HRP), an enzyme that catalyses oxidation reactions with hydrogen peroxide. When exposed to visible light, the hydrophobic triene-enol functionality switches to a more polar cyclopentenone, causing an increase in membrane permeability. Hydrogen peroxide and pyrogallol were introduced to the exterior solution which then permeated through the polymersome membrane, resulting in a higher rate of pyrogallol oxidation in the presence of HRP.

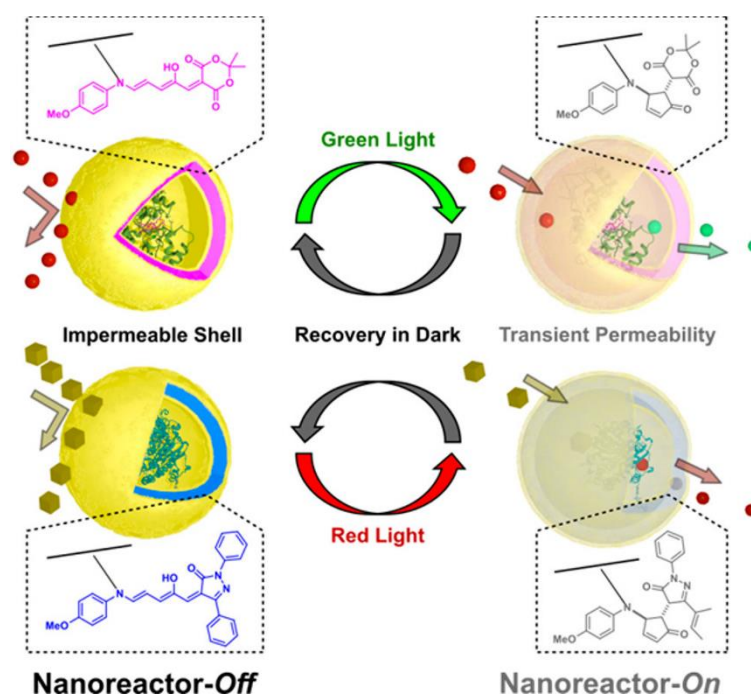


Figure 4.2. DASA-functionalized visible light-responsive nanoreactors. Adapted from Rifaie-Graham *et al.*⁸

Polymersomes with encapsulated cargo have also been shown to be effective methods for therapeutic applications.⁹ As an example, Huh and Haam prepared biodegradable polymersomes from poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (PEG-*b*-PLA) block copolymers with dual encapsulation of short interfering RNA (siRNA) and doxorubicin (DOX)

(Figure 4.3).¹⁰ The authors demonstrated steady release of both cargos over the timescale of the experiment, as well as increased cytotoxic efficacy compared with free DOX.

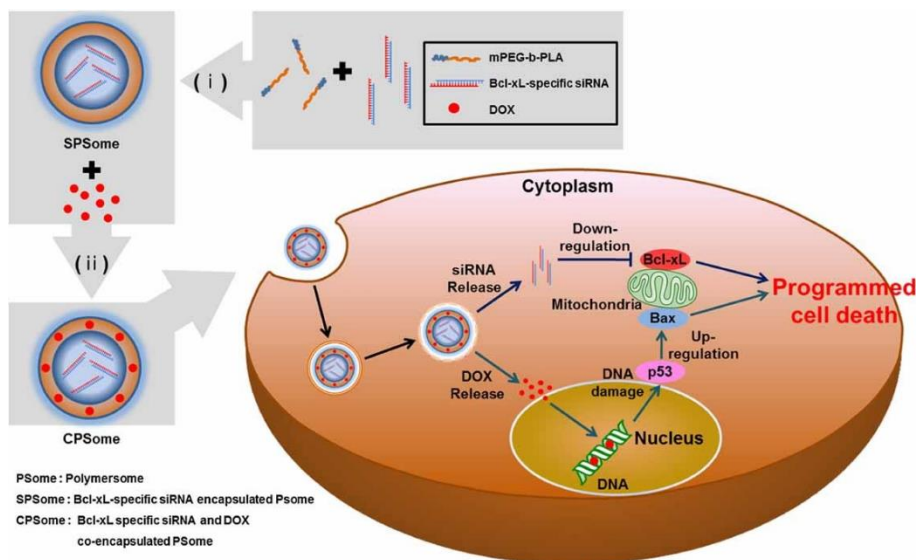


Figure 4.3. Schematic illustration of the co-delivery system using biodegradable polymersomes to deliver siRNA and DOX. Adapted by Kim *et al.*¹⁰

The properties associated with polymersomes make them excellent candidates for use as catalytic nanoreactors, drug delivery systems, cellular models, and nanosensors.¹¹⁻¹⁴ However, the encapsulation efficiency of water-soluble cargos is largely influenced by the mechanism of polymersome formation.¹⁵

4.2.2 Polymersome mechanism of formation

Two mechanisms have been proposed for polymer self-assembly to produce vesicles. The first, termed Mechanism I, starts with a homogenous mixture of amphiphilic molecules which aggregate to form micelles. The micelles then grow into bilayer sheets which eventually undergo bending and close to form vesicles (Figure 4.4, top). This mechanism has been widely reported for a variety of amphiphilic molecules, including block copolymers, both experimentally¹⁶⁻¹⁸ and computationally.¹⁹⁻²²

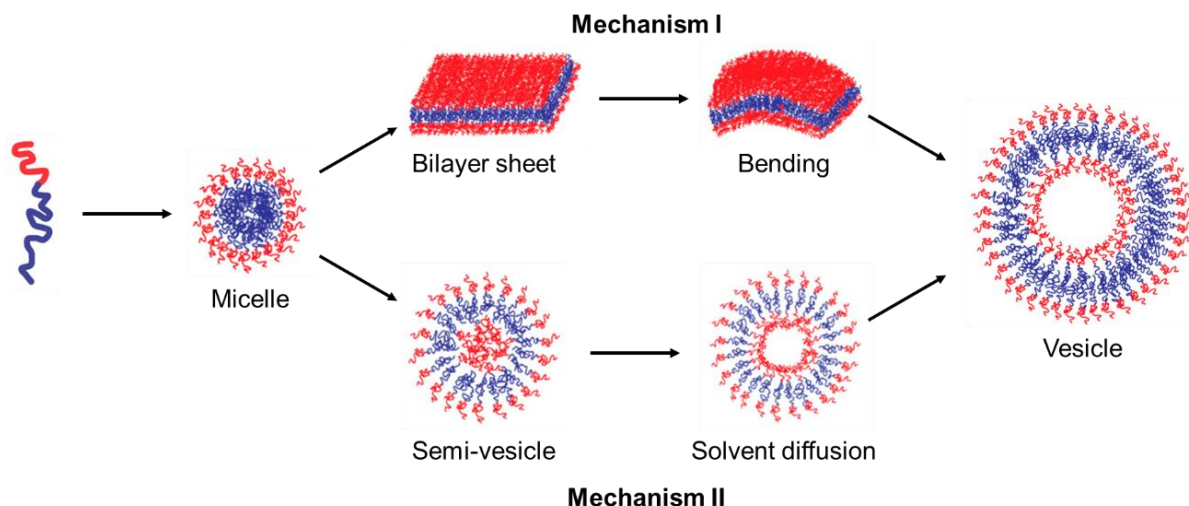


Figure 4.4. Schematic representation of the two proposed mechanisms for vesicle formation: mechanism I and mechanism II. Adapted from Iqbal *et al.*⁹

Mechanism II also starts with spherical micelle formation. However, micelles continue to grow until there is a rearrangement of some of the unimers (flip-flop) forming a semi-vesicle (a vesicle with no lumen). Solvent molecules then diffuse through the bilayer membrane resulting in the formation of a lumen (Figure 4.4, bottom). Mechanism II is rarely reported experimentally²³ but has been demonstrated computationally using the external potential dynamics (EPD) method.^{24, 25}

The mechanism by which block copolymer nano-objects form will influence their ability to encapsulate water-soluble cargos within their internal lumens. In mechanism I, the solution in which self-assembly is occurring will get trapped during closure of the bilayer sheet, resulting in encapsulation of any molecules contained in solution. This ability has been widely applied to a number of cargos, from simple fluorescent molecules to more complex biological therapeutics.^{9, 26, 27}

In contrast, encapsulation of water-soluble molecules within a polymersome lumen is impossible by mechanism II without an external stimulus or driving force. For example, when a concentration gradient is applied, cargo can diffuse through the membrane bilayer and become encapsulated. However, once the concentration gradient is reversed (i.e. removal of externally located cargo), cargo will permeate out of the polymersome. This leakage could be overcome if the membrane permeability of the polymersome could be tuned. Cargo could be allowed to passively diffuse inside the polymersome by application of a positive concentration gradient whilst membrane permeability was high. Then, following a shift to low membrane permeability, the cargo would be retained when the concentration gradient is reversed.

In this chapter we report on the encapsulation of hydrophilic cargo using nano-objects prepared by ROMPISA. The impact of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), a pH-sensitive, fluorescent dye, on both polymerisation and self-assembly behaviour was determined. A range of techniques were tested for their efficacy in nano-object purification and the impact of HPTS concentration on encapsulation efficiency was explored. Complementary to this, the encapsulation of inorganic nanoparticles with the aim of determining the location of cargo within the nano-object was investigated using dry-state TEM. Finally, the mechanism of formation for a model ROMPISA system was probed using time-resolved SAXS and provided tentative evidence that ROMPISA nano-objects form *via* an alternative mechanism to that seen in other PISA systems (e.g. RAFT PISA).

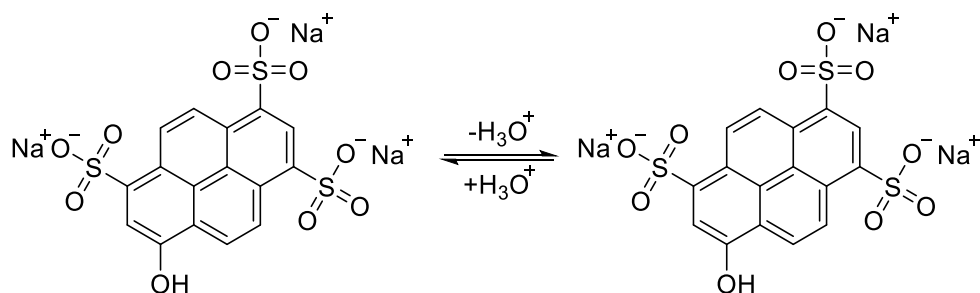
4.3 Results and Discussion

4.3.1 Determining the encapsulation efficiency of a small molecule fluorophore within nano-objects synthesised *via* aqueous ROMPISA

Previously, our group has reported the preparation of a range of norbornene-derived diblock copolymer nano-objects by aqueous ROMPISA.²⁸ However, to date, the encapsulation efficiency of these nano-objects is yet to be reported and would provide insight into their effectiveness in various applications, such as nanoreactors, drug delivery systems and cellular models.

4.3.1.1 Encapsulation of 8-hydroxypyrene-1,3,6-trisulfonic acid within ROMPISA nano-objects

8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) is a pH-sensitive, fluorescent dye that is soluble in water at all pH values. In solution, HPTS dissociates to its phenate form, the extent of which depends on pH ($pK_a = 7.2$) (Scheme 4.1).²⁹ When irradiated at 450 nm, the phenate form of HPTS forms a charge-transfer state that can undergo a proton-transfer reaction, producing a fluorescence emission at 510 nm.³⁰ At low pH, the relative concentration of the phenate form is low, resulting in a low fluorescence emission intensity. As pH increases, the ionization equilibrium shifts to the phenate form, leading to a higher fluorescence intensity (Figure 4.5).



Scheme 4.1. Ionisation equilibrium of HPTS to its phenate form.

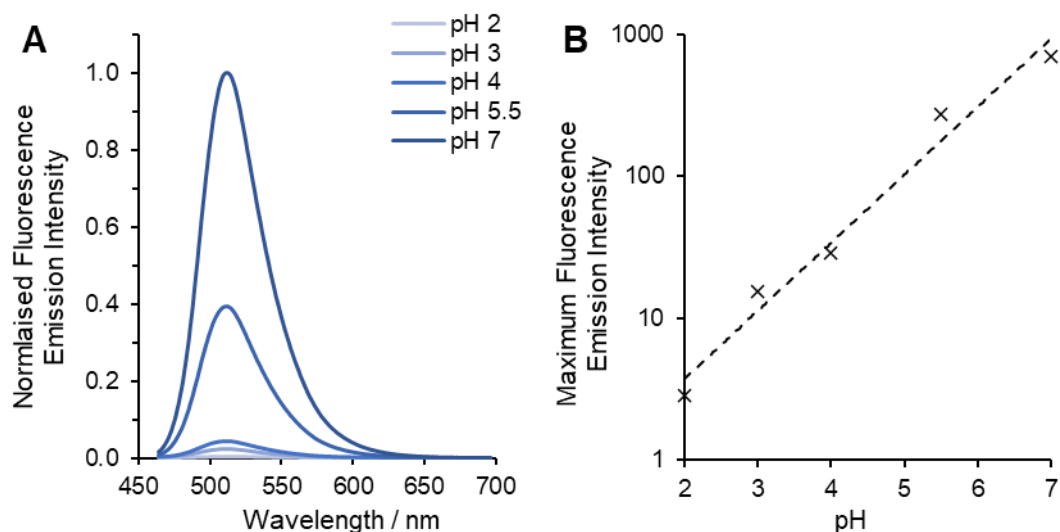
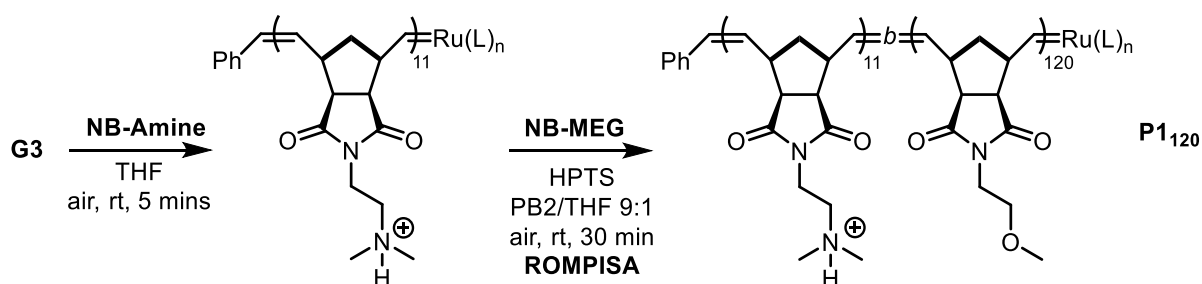


Figure 4.5. (A) Normalised fluorescence emission spectra of 10 mM HPTS at different solution pH. (B) pH calibration curve for 10 mM HPTS.

To explore the encapsulation ability of ROMPISA polymersomes, the amphiphilic block copolymers, **P1₁₂₀**, **P(NB-Amine)₁₁-b-P(NB-MEG)₁₂₀**, containing a corona formed from **NB-Amine** and a core of **NB-MEG** (DP = 120) were prepared in the presence of HPTS (5 mg mL⁻¹) (Scheme 4.2). This was performed analogously to diblock polymersomes synthesised in Chapter 3, Section 3.3.3.1. **P1₁₂₀** polymersomes were also prepared with no HPTS present as a control.



Scheme 4.2. Synthesis of **P1₁₂₀** diblock copolymer in the presence of HPTS by ROMPISA.

An increase in turbidity was observed for **P1₁₂₀** solution that contained no HPTS. However, precipitation was observed for **P1₁₂₀** with HPTS, suggesting the polymersomes were no longer

colloidally stable in aqueous solution. SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.3$) for **P1₁₂₀** solutions both with and without HPTS present (Table 4.1 and Figure 4.6). This result demonstrates that HPTS had no impact on the polymerisation of the diblock copolymer.

Table 4.1. Characterisation summary of **P1₁₂₀**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\mathcal{D}_{GPC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}
P(NB-Amine) ₁₁	3.4	3.2	1.13	N/A	N/A
P1₁₂₀ , without HPTS	29.9	44.3	1.26	77	0.25
P1₁₂₀ , with HPTS	29.9	50.6	1.23	1537	0.94

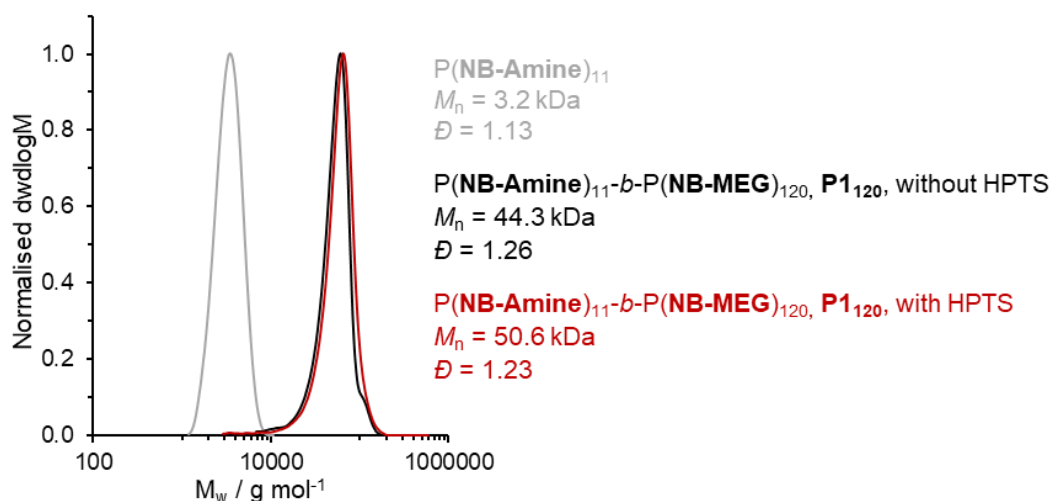
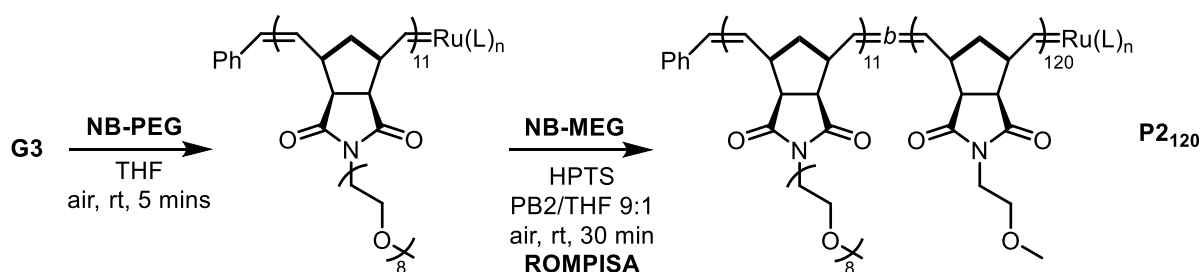


Figure 4.6. Normalised SEC traces (THF eluent, PMMA standards) of **P1₁₂₀**, with (red) and without (black) HPTS, and intermediate polymers.

The resultant solutions were analysed by DLS which showed an average hydrodynamic diameter of 77 nm for the native particles without HPTS present during self-assembly. However, the HPTS sample revealed a multimodal distribution (Table 4.1 and Figure S4.1). It

is clear to see that HPTS disrupts the self-assembly process of ROMPISA for **P1₁₂₀** polymersomes. It is suspected that the HPTS associates with the amine groups, reducing solvation of the corona block and causing precipitation.

To avoid the precipitation observed when using **NB-Amine**, the corona-forming monomer was changed to uncharged **NB-PEG**. As such, diblock copolymer **P2₁₂₀**, P(**NB-PEG**)_{11-*b*}-P(**NB-MEG**)₁₂₀, was synthesised, with and without HPTS (5 mg mL⁻¹), *via* aqueous ROMPISA (Scheme 4.3).

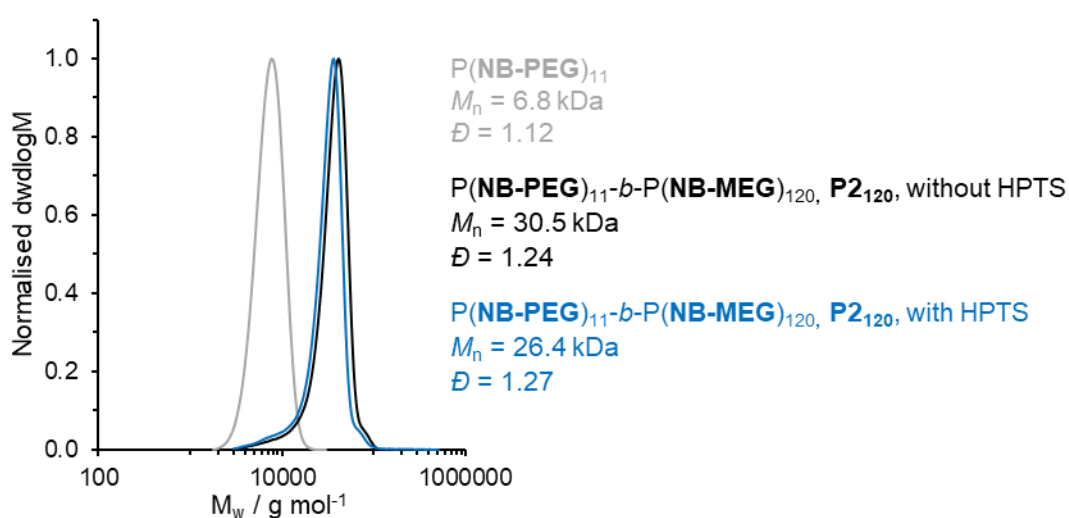


Scheme 4.3. Synthesis of **P2₁₂₀** diblock copolymer in the presence of HPTS by ROMPISA.

Both **P2₁₂₀** solutions (with and without HPTS) showed an increase in turbidity but no precipitation was observed. SEC analysis of both polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.3$), with HPTS once again having no impact on the resulting molecular weight or dispersity of ROMPISA diblock copolymers (Table 4.2 and Figure 4.7).

Table 4.2. Characterisation summary of **P2₁₂₀**.

Polymer	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\bar{D}_{GPC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}	L_{TEM} / nm
P(NB-PEG) ₁₁	6.6	6.8	1.12	N/A	N/A	N/A
P2₁₂₀ , without HPTS	33.1	30.5	1.24	52	0.24	47 ± 6
P2₁₂₀ , with HPTS	33.1	26.4	1.27	48	0.21	41 ± 7

**Figure 4.7.** Normalised SEC traces (THF eluent, PMMA standards) of **P2₁₂₀**, with (blue) and without (black) HPTS, and intermediate polymers.

DLS analysis showed average hydrodynamic diameter of 48 and 52 nm with and without HPTS present, respectively (Table 4.2 and Figure 4.8). DLS traces showed a dominant peak at 50 nm and a secondary peak at > 100 nm for both solutions. TEM analysis showed spherical polymersomes with similar average diameters (41 and 47 nm for with and without HPTS, respectively) for both solutions of **P2₁₂₀** confirming that HPTS had little impact on the self-assembly of uncharged diblock copolymer nano-objects (Table 4.2 and Figure 4.9).

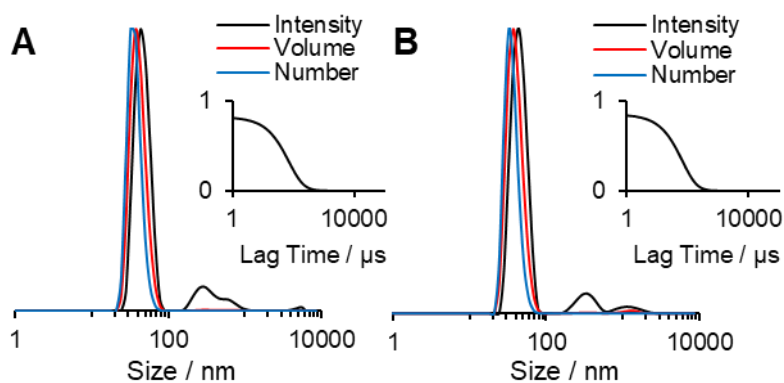


Figure 4.8. Normalised DLS analysis of **P2₁₂₀** (A) without and (B) with HPTS.

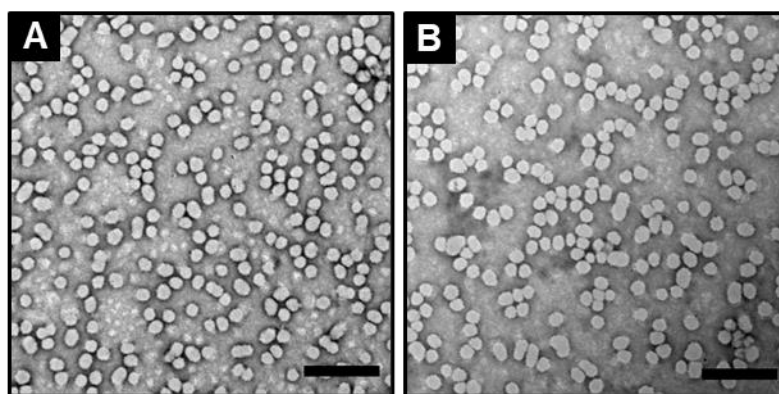


Figure 4.9. Dry-state TEM images of **P2₁₂₀** nano-objects (A) without and (B) with HPTS. Scale bar = 200 nm.

HPTS containing **P2₁₂₀** solution was then diluted and analysed by fluorescence spectroscopy. When irradiated at 450 nm, an emission peak was observed at 510 nm, as expected for HPTS in a low pH aqueous solution (Figure 4.10, black).³¹

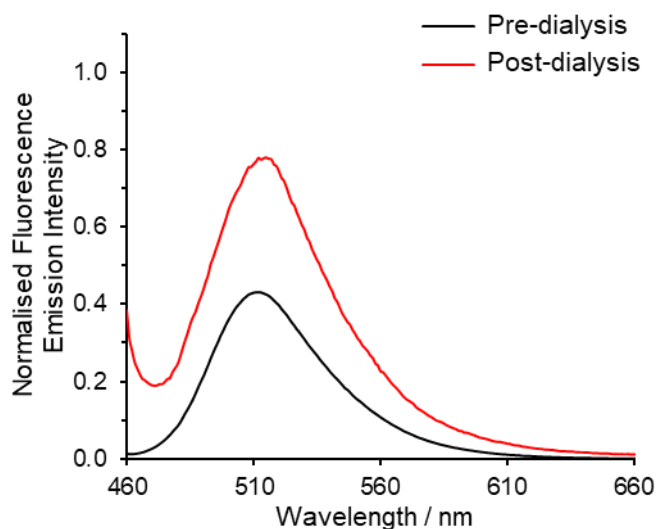


Figure 4.10. Normalised fluorescence emission spectra of **P2₁₂₀** before (black) and after (red) dialysis against H₂O.

4.3.1.2 Purification of HPTS from ROMPISA nano-objects

Unencapsulated dye was removed from solution by dialysis against H₂O, incorporating at least three water changes, and then analysed again by fluorescence spectroscopy (Figure 4.10, red). The fluorescence emission spectrum of **P2₁₂₀** after dialysis showed a fluorescence emission peak at 510 nm, consistent with fluorescent emission from the phenate form of HPTS. However, the emission intensity for dialysed **P2₁₂₀** was greater after dialysis, suggesting that the pH of the internal lumen was increased during the dialysis purification. To maintain the internal lumen pH, the dialysis solution was changed to a pH 2 solution.

Unencapsulated dye was removed from solution by dialysis against a HCl solution (pH 2), incorporating at least three solution changes, and then analysed again by fluorescence spectroscopy (Figure 4.11, red). Following dialysis at pH 2, **P2₁₂₀** showed an emission intensity of 2.0×10^{-3} at 512 nm. When compared to the emission intensity prior to dialysis (Figure 4.11,

black), this is equivalent to an uptake percentage of 0.20%, a much lower value than seen in other polymersome systems.³²

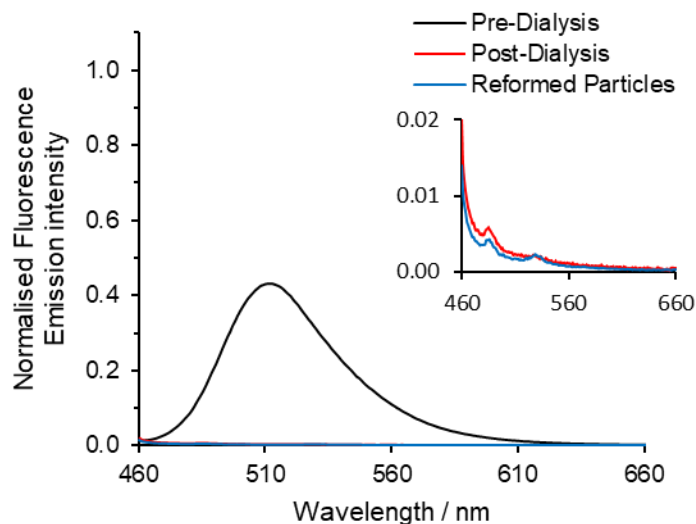


Figure 4.11. Normalised fluorescence emission spectra of **P2₁₂₀** before (black) and after (red) dialysis at pH 2 and **P2₁₂₀** after being disassembled and reformed (blue).

Initially, it was suspected that the self-assembled structure may have collapsed during dialysis, resulting in the encapsulated dye leaking out into the exterior solution and reducing the emission intensity observed. As the nano-objects are initially formed under kinetic control, disassembly and reassembly of the diblock copolymers results in the formation of the thermodynamically favoured structures – large polymersomes with diameters above 200 nm.³³ However, DLS and TEM analysis showed no change and suggests dialysis had no effect on the nano-object self-assembly (Table S4.1 and Figure S4.2 and S4.3).

Alternatively, we theorised that the encapsulated environment of the HPTS dye was affecting its fluorescence intensity. HPTS has been shown to undergo aggregate induced fluorescence quenching by forming a stable ground state complex which decreases the efficiency of the excited-state proton-transfer reaction.³⁴ As such, disassembly of the particles would release

the encapsulated HPTS into the surrounding solution, reducing the local concentration and preventing aggregates from forming.

As such, THF was added to **P2₁₂₀** nano-objects and stirred for 30 minutes to ensure full chain dissolution. THF was then removed under vacuum and the diblock copolymers resuspended in PB2 buffer. However, no change was seen in the fluorescence emission spectrum for the reformed particles (Figure 4.11 (blue)). This suggests HPTS may have leaked out from the internal lumen of the ROMPISA nano-objects during purification. Dialysis is a slow purification technique, requiring multiple solution changes over several days, increasing the chance of loss of encapsulated material. As such, investigation into faster purification techniques is of interest.

Two other common methods of small molecule purification are spin filtration and gel filtration chromatography. Like dialysis, both techniques are a method of size exclusion, but are not restricted by the rate of small molecule diffusion. As such, these techniques are a more convenient and quicker alternative to dialysis.

P2₁₂₀ nano-objects formed in the presence of HPTS were purified by either spin filtration or gel filtration chromatography. Following spin filtration, DLS analysis of the resultant solution showed a multimodal distribution with an average hydrodynamic diameter of 250 nm, an increase of 500% from the original particles (Figure S4.4). From this, we conclude that spin filtration significantly disrupts the self-assembly of ROMPISA nano-objects and is therefore an unsuitable purification method.

Following purification by gel filtration chromatography, **P2₁₂₀** particles showed a unimodal distribution with an average hydrodynamic diameter of 62 nm by DLS (Figure 4.12). TEM

analysis indicated spherical nano-objects of similar size to the original particles (43 nm) were eluted from the column (Figure 4.13). This suggests purification by gel filtration chromatography had little impact on the self-assembled structure of the ROMPISA nano-objects.

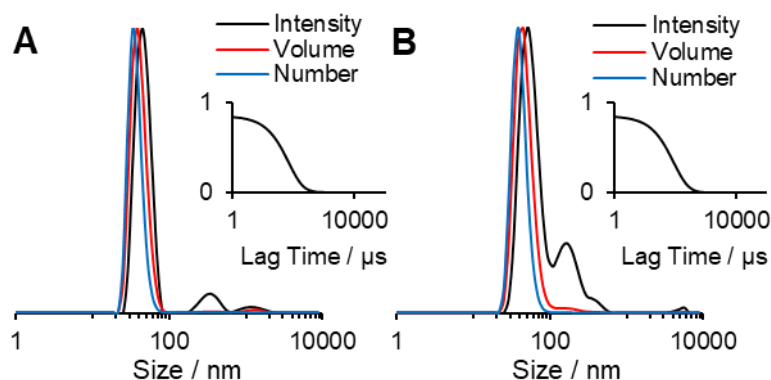


Figure 4.12. Normalised DLS analysis of **P2₁₂₀** (A) before and (B) after purification by gel filtration chromatography.

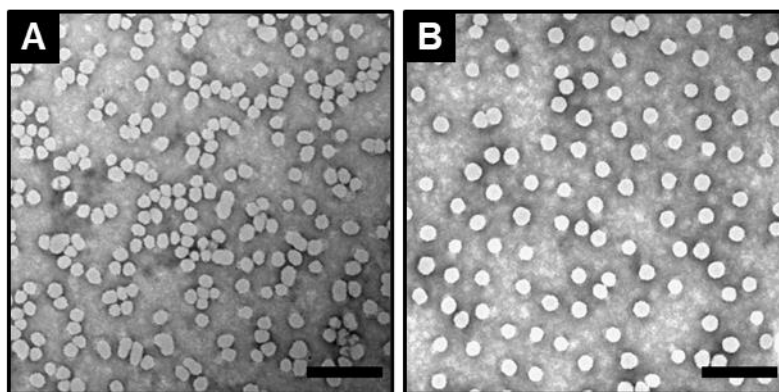


Figure 4.13. Dry-state TEM images of **P2₁₂₀** nano-objects (A) before and (B) after purification by gel filtration chromatography. Scale bar = 200 nm.

Fluorescence spectroscopy of **P2₁₂₀** following purification by gel filtration chromatography showed a fluorescence emission intensity ($\lambda_{Em} = 510$ nm) that was 0.24% of the maximum emission intensity obtained prior to purification (Figure 4.14). This value is very similar to that measured after dialysis, even though purification by gel filtration chromatography is much

faster than that of dialysis (< 10 mins vs. 2-3 days, respectively). Therefore, it was concluded that, rather than losing encapsulated material, low fluorescence emission intensity of purified **P2₁₂₀** is due to a low concentration of HPTS encapsulated in the initial self-assembly process.

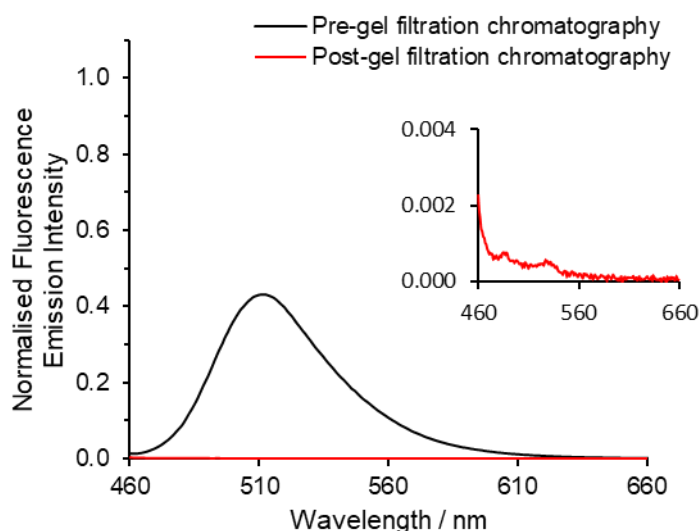


Figure 4.14. Normalised fluorescence emission spectra of **P2₁₂₀** before (black) and after (red) purification by gel filtration chromatography.

4.3.1.3 Concentration dependence on encapsulation efficiency of HPTS

To increase the concentration of HPTS encapsulated within ROMPISA nano-objects, and thus increase the fluorescence emission intensity, a greater concentration of HPTS was used during nano-objects self-assembly. **P2₁₂₀** diblock copolymer nano-objects were prepared as before with 0, 1, 10 or 100 mg mL⁻¹ HPTS added during the second step of the reaction.

DLS analysis showed monomodal size distributions for solutions of 1 mg mL⁻¹ HPTS and no HPTS (Table 4.3 and Figure 4.15). However, when the concentration of HPTS present in solution was increased to 10 and 100 mg mL⁻¹, the presence of larger nano-objects (> 100 nm) was observed. This was shown to be more prominent at the highest concentration of HPTS and could be due to either aggregation of HPTS or an interference in the self-assembly of **P2₁₂₀**

nano-objects at high concentrations of HPTS. Average hydrodynamic diameters increased from 62 to 71 and finally to 80 nm as the concentration of HPTS was increased from 1 to 10 and then to 100 mg mL⁻¹, respectively.

Table 4.3. Characterisation summary of **P2₁₂₀** synthesised with different concentrations of HPTS.

[HPTS] / mg mL ⁻¹	$Z_{\text{avg,DLS}}$ / nm	PDI _{DLS}
0	52	0.24
1	62	0.25
10	71	0.24
100	80	0.25

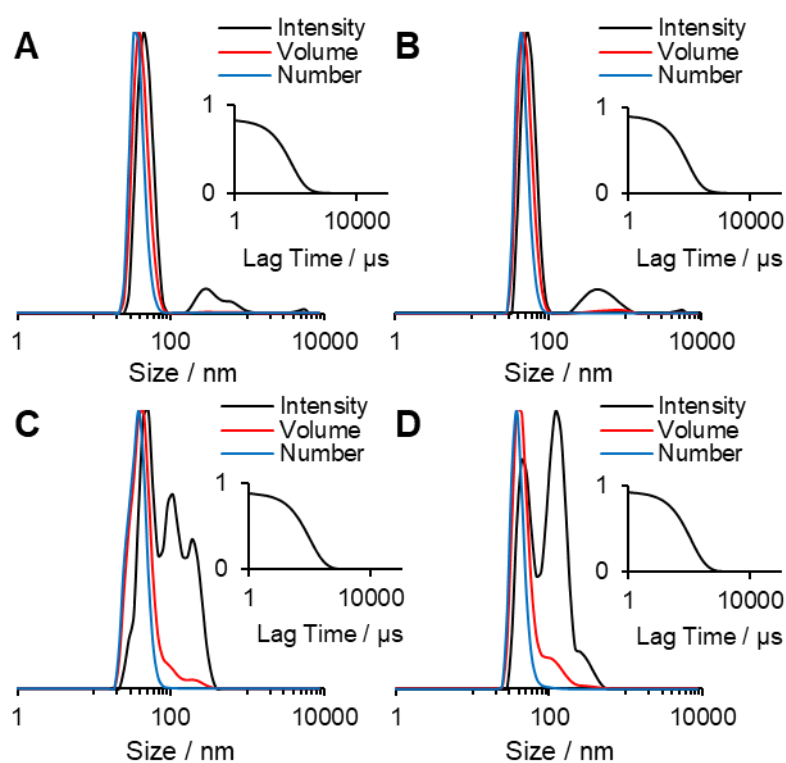


Figure 4.15. Normalised DLS analysis of **P2₁₂₀** synthesised at different concentrations of HPTS (A) 0, (B) 1, (C) 10 and (D) 100 mg mL⁻¹.

As expected, prior to purification, fluorescence spectroscopy of the **P2₁₂₀** solutions showed decreasing emission intensity as the concentration of HPTS was decreased (Table S4.4 and Figure 4.16).

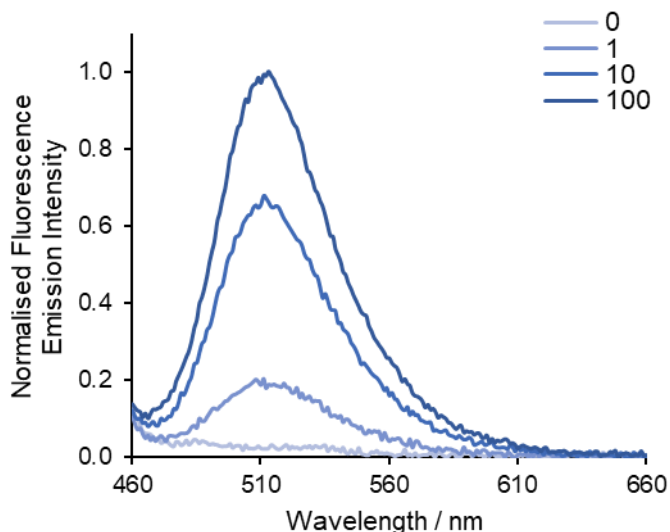


Figure 4.16. Normalised fluorescence emission spectra of **P2₁₂₀** synthesised at different concentrations of HPTS (A) 0, (B) 1, (C) 10 and (D) 100 mg mL⁻¹.

Unencapsulated dye was removed from solution by dialysis against a HCl solution (pH 2), incorporating at least three solution changes, and then analysed again by fluorescence spectroscopy (Figure 4.17). Following dialysis at pH 2, **P2₁₂₀** showed emission intensities of 0.9×10^{-3} , 1.3×10^{-3} , and 2.8×10^{-3} (normalised to the original fluorescence intensities) for 1, 10 and 100 mg mL⁻¹ at 512 nm, respectively.

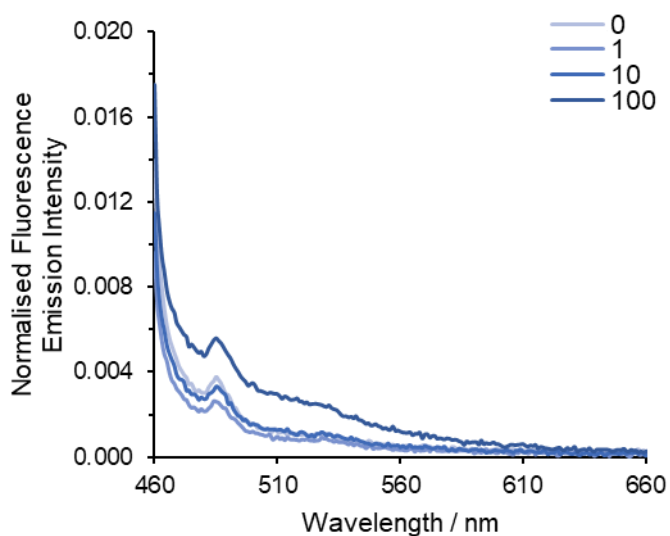


Figure 4.17. Normalised fluorescence emission spectra of **P2₁₂₀** synthesised at different concentrations of HPTS following dialysis at pH 2 (A) 0, (B) 1, (C) 10 and (D) 100 mg mL⁻¹.

The control sample, for which there was no HPTS present in solution during self-assembly, had an emission intensity of 1.2×10^{-3} , indicating the fluorescence emission detected for **P2₁₂₀** solutions is within the error of background noise. As such, increasing the concentration of HPTS in solution during self-assembly did not increase the concentration of encapsulated HPTS within ROMPISA nano-objects.

4.3.2 Encapsulation of inorganic nanoparticles into polymeric nano-objects synthesised *via* aqueous ROMPISA

As encapsulation of a small molecule fluorophore was not observed, encapsulation of inorganic nanoparticles and analysis by TEM was attempted. This is a complementary technique to bulk measurements (e.g. fluorescence spectroscopy) for assessing encapsulation as you cannot determine encapsulation efficiency by TEM alone. However, clear distinction can be made between the membrane and internal lumen for most nano-objects allowing for the location of encapsulation to be elucidated.

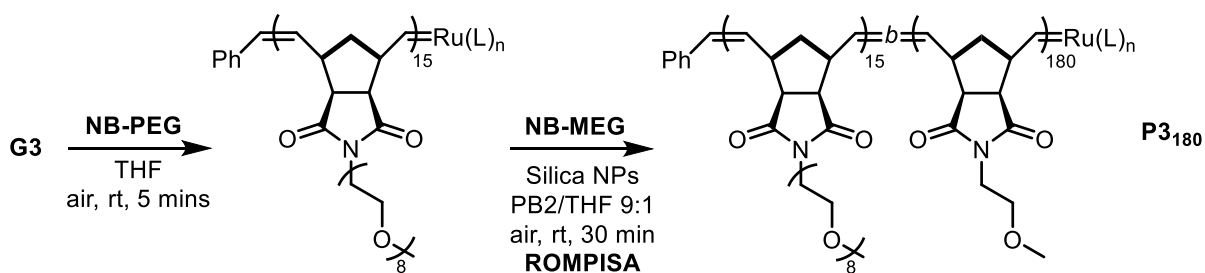
For TEM, as the electrons pass through the sample, they are scattered by the electrostatic potential of the constituent elements in the specimen. As such, the beam interacts greatest with elements of higher atomic number, allowing different elements to be identified. To assess the encapsulation ability of ROMPISA nano-objects by TEM, the cargo material must be of a higher atomic number than those found in the diblock copolymers: in this case, hydrogen, carbon, nitrogen, and oxygen.

In addition to moving to inorganic nanoparticles, the corona DP of the ROMPISA nano-objects was also lengthened from 11 to 15 units and the core DP increased to 180. Firstly, this would prevent any fusion of ROMPISA nano-objects as observed in previous reports.³³ Secondly, a longer corona increases the stabilising ability of the block, leading to the formation of larger nano-objects.

4.3.2.1 Silica nanoparticles

Silica nanoparticles (NPs) are both commercially available in the form of concentrated dispersions and possess sufficient electron contrast to allow their visualization by TEM. As such, they have been used as a model cargo for encapsulation within polymer nano-objects.³⁵

With the aim of encapsulating silica NPs, diblock copolymer **P3₁₈₀**, P(NB-PEG)₁₅-*b*-P(NB-MEG)₁₈₀, was synthesised, in the presence of silica NPs at different weight percentages, *via* aqueous ROMPISA (Scheme 4.4). Two control experiments were also performed: (1) **P3₁₈₀** nano-objects were prepared with no silica present and (2) **P3₁₈₀** nano-objects were prepared, and silica nanoparticles were added after synthesis.



Scheme 4.4. Synthesis of **P3₁₈₀** diblock copolymer in the presence of silica NPs by ROMPISA.

SEC analysis of **P3₁₈₀** polymers without silica present showed a unimodal molecular weight distribution and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.2$) (Table S4.5 and S4.7 and Figure 4.18). With silica in solution during ROMPISA, SEC analysis of **P3₁₈₀** indicates a slight increase in M_n and a small portion of macroinitiator chains not being extended. This may be caused by the macroinitiator permeating into the pores of the silica NPs, preventing further chain extension.

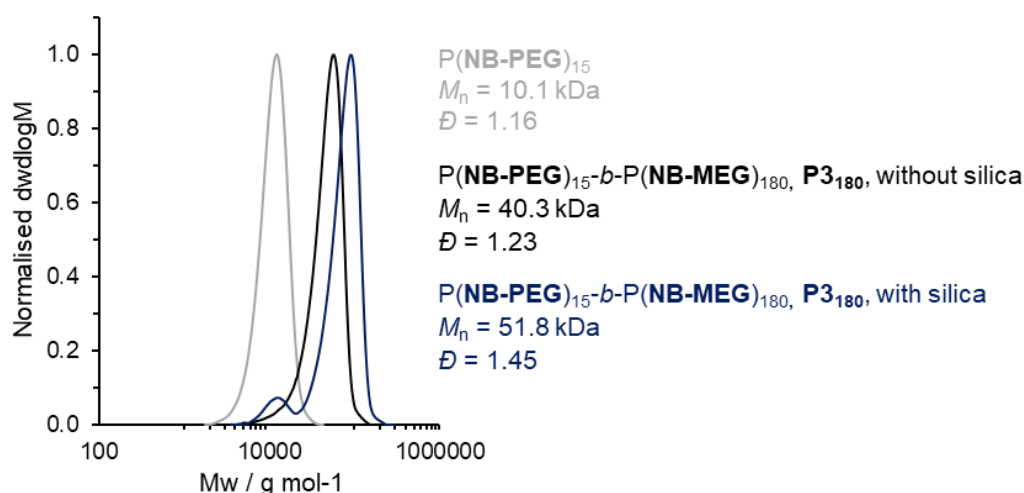


Figure 4.18. Normalised SEC traces (THF eluent, PMMA standards) of P3_{180} , with (blue) and without (black) silica NPs, and intermediate polymers.

DLS analysis showed a bimodal distribution with an average hydrodynamic diameter of 106 nm for P3_{180} without silica present (Figure S4.7). In contrast, multimodal distributions were observed for all samples containing silica, regardless if they were added before or after self-assembly. This suggests a significant interaction between the silica nanoparticles and the ROMPISA nano-objects.

TEM analysis showed spherical nano-objects with average diameter 64 nm for P3_{180} without silica present (Figure 4.19). For samples containing silica, large, low-contrast spherical nano-objects with similar diameter to native P3_{180} were observed surrounded by smaller, high-contrast spherical particles.

No encapsulation of silica nanoparticles was observed by TEM. Instead, it was theorised that the PEG stabilising block of P3_{180} nano-objects was interacting with the silica NPs through hydrogen bonding, leading to them adhering to the surface of the ROMPISA particles.³⁶

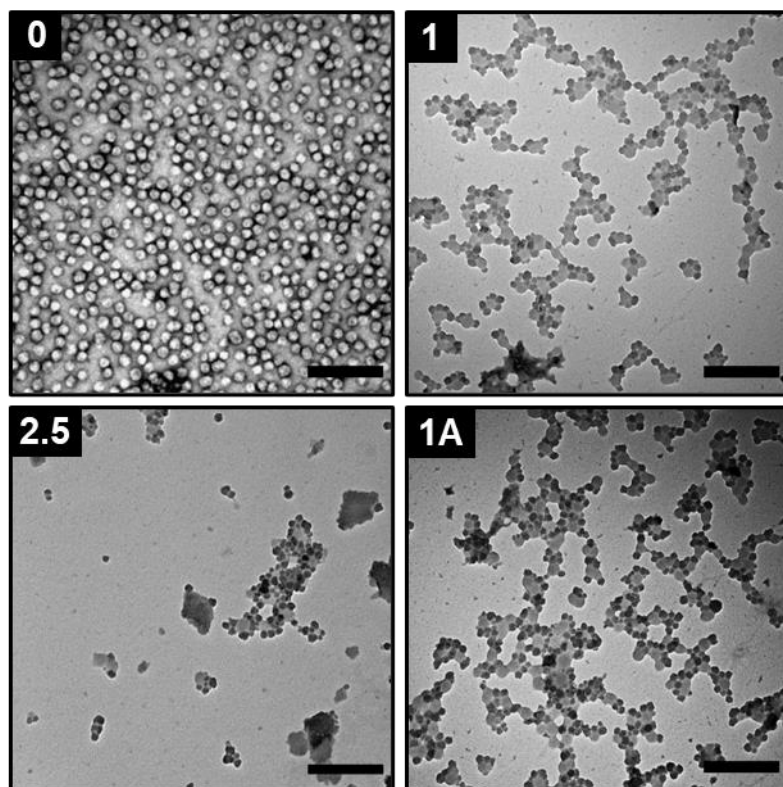


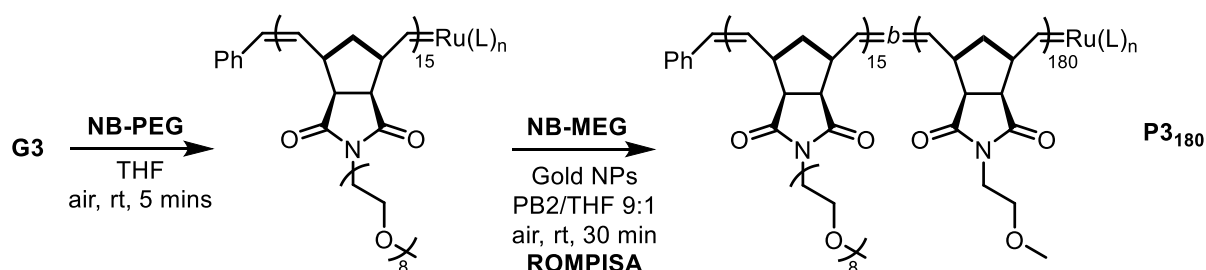
Figure 4.19. Dry-state TEM images of native $P3_{180}$ particles (UA staining); $P3_{180}$ particles synthesised in the presence of 1 wt% silica (1), 2 wt% silica (2), and without silica present but added after self-assembly (1A) (no staining). Scale bar = 200 nm.

In addition, the size of the silica NPs (Table S4.6) means there is a large entropic barrier to encapsulation due to the resulting restriction of particle movement within the nano-object. As such, NPs with a smaller diameter should have a lower entropic barrier to encapsulation within ROMPISA nano-objects.

4.3.2.2 Gold nanoparticles

Gold NPs have previously been used to demonstrate proof of encapsulation by direct visualisation.³⁷⁻⁴⁰ These nanoparticles can be as small as 5 nm with very low dispersity whilst also possessing excellent contrast for TEM measurements.

Analogous to silica, diblock copolymer **P3₁₈₀**, **P(NB-PEG)₁₅-*b*-P(NB-MEG)₁₈₀**, was synthesised, in the presence of citrate-stabilised gold NPs at different concentrations, *via* aqueous ROMPISA (Scheme 4.5).



Scheme 4.5. Synthesis of **P3₁₈₀** diblock copolymer in the presence of gold NPs by ROMPISA.

DLS analysis showed bimodal distributions for **P3₁₈₀** in the presence of gold NPs with average hydrodynamic diameters of between 61 and 110 nm (Table S4.9 and Figure 4.20). The peak above 100 nm was observed in all samples, including native ROMPISA nano-objects and gold NPs in isolation. This is likely due to aggregation of either gold NPs, ROMPISA nano-objects or a combination of the two. This result suggests the presence of gold nanoparticles had little impact on the self-assembly process in ROMPISA.

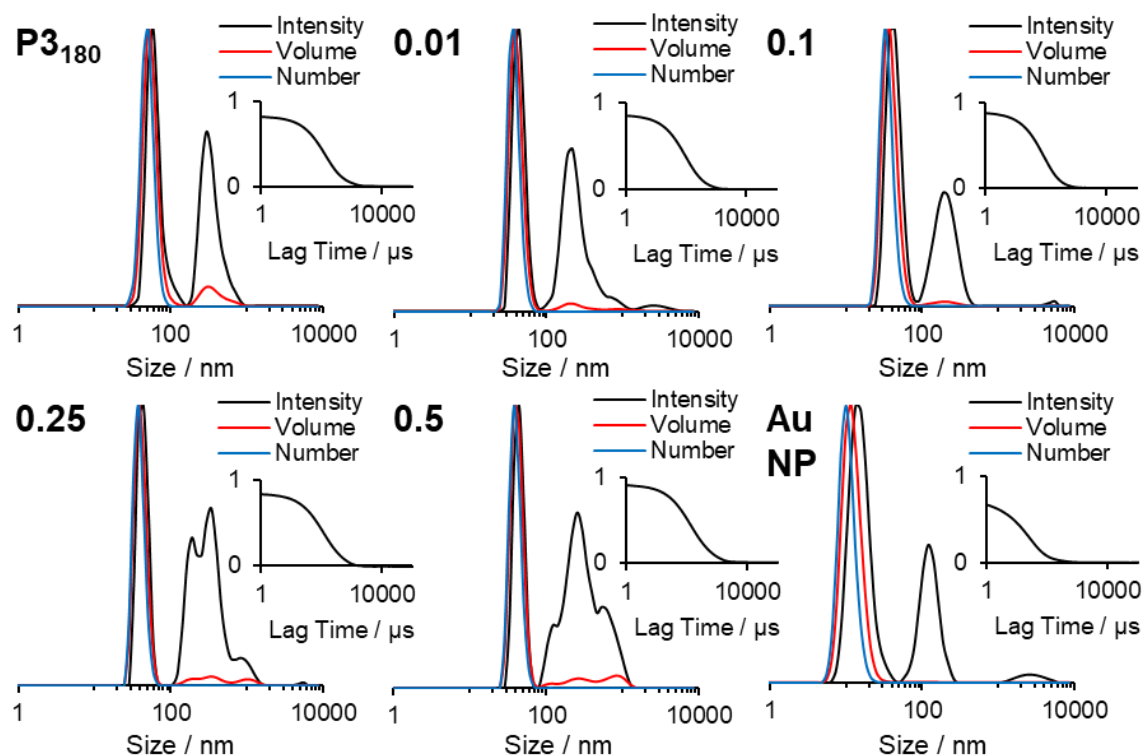


Figure 4.20. Normalised DLS analysis of native **P3₁₈₀** particles; **P3₁₈₀** particles synthesised in the presence of different concentrations of gold NP (0.01 refers to the optical density (OD) of gold NP in solution); and gold nanoparticles (OD = 0.5).

TEM analysis showed spherical nano-objects with average diameter 4 nm for gold NPs. (Figure 4.21). For **P3₁₈₀** samples containing gold NPs, spherical nano-objects with average diameter 67 nm were observed, consistent with native **P3₁₈₀** nano-objects. This provides further evidence that gold NPs do not impact the self-assembly process.

However, direct visualisation of both ROMPISA nano-objects and gold NPs was only possible at the highest concentration of gold NPs (optical density (OD) = 0.5). In this case, it was observed that small, dark spherical objects were surrounding larger, spherical objects which have lower contrast. Similarly to the experiment using silica NPs, it was concluded that no successful encapsulation of gold NPs within ROMPISA nano-objects had occurred. In addition, the drying effect of TEM analysis may have caused aggregation to the **P3₁₈₀** nano-objects.

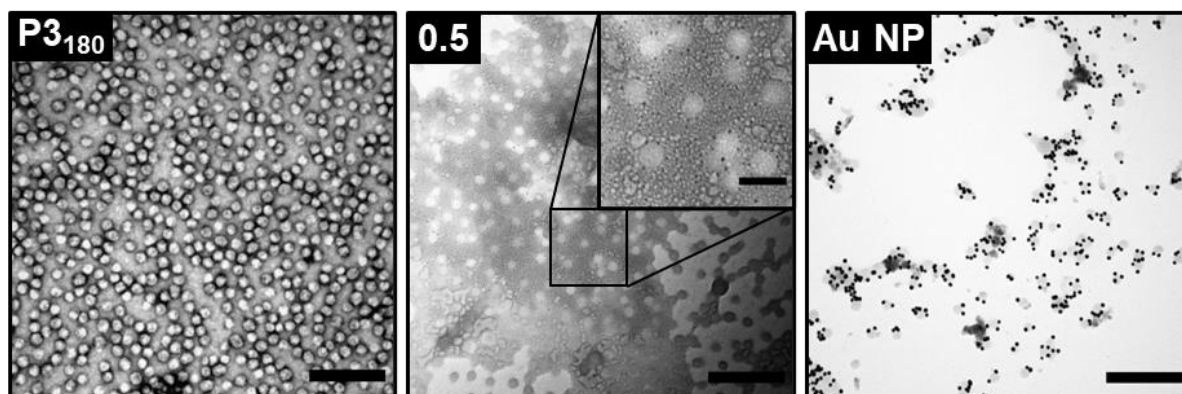


Figure 4.21. Dry-state TEM images of native **P3₁₈₀** particles (UA staining); **P3₁₈₀** particles synthesised in the presence of gold NP (OD = 0.5), and gold nanoparticles (OD = 0.5) (no staining). Scale bar = 200 nm.

In summary, there was no evidence to support the theory of successful encapsulation of cargo within ROMPISA nano-objects. A similar result was previously reported by Adams *et al.*²³ The authors demonstrated that polymersomes formed from a poly(ethylene glycol)-block-poly(N,N-diethylaminoethyl methacrylate) (PEG-*b*-PDEAMA) copolymer by pH switch were unable to encapsulate either rhodamine B or riboflavin during polymersome formation, although internalisation of riboflavin was possible when left for a longer incubation time, likely due to the uncharged dye diffusing through the polymer membrane.

Further analysis by cryo-TEM revealed the presence of intermediate structures, which the authors classified as semi-vesicles. This result, alongside the inability to encapsulate water-soluble cargos, indicates that the vesicles in this report are formed differently to the standard mechanism of spheres to worms to vesicles observed in RAFT PISA.^{18, 41}

ROMPISA nano-objects contain rigid polymer chains that adopts a rod-like conformation to give glassy membranes. Therefore, a morphology transition from spheres to vesicles *via* an enclosed bilayer may be too high in energy due to the requirement for membrane flexibility. As such, it was postulated that the ROMPISA systems may be proceeding *via* mechanism II.

4.3.3 Investigating the mechanism of formation of nano-objects synthesised *via* aqueous ROMPISA using small angle x-ray scattering

As shown earlier in this chapter, encapsulation of cargo, either a small molecule fluorophore or inorganic NP, within ROMPISA nano-objects was not observed. As such, it was theorised that block copolymer nano-objects prepared by ROMPISA are forming under mechanism II, in which unimers flip-flop to form semi-vesicles and solvent diffusion results in the formation of an internal lumen.

A key technique used to determine the mechanism of formation for block copolymer nano-objects is TEM, allowing for direct visualisation of intermediate morphologies.¹⁸ This method requires either a sufficiently long polymerisation time or efficient quenching mechanisms that do not disrupt the self-assembled structure. However, ROMPISA nano-objects have been shown to reach 99% conversion in 30 mins, with initial self-assembled structures observed as quickly as 20 seconds after the start of the PISA step.³³

In addition, ROMP is typically quenched using a small, alkene-functionalised molecule, such as ethyl vinyl ether (EVE), which cleaves the active Ru initiator from the growing polymer chain and deactivates the metal centre to form a Fischer carbene, preventing further propagation. However, ROMPISA nano-objects are formed under kinetic control and so adding plasticisers gives a more stable structure.³³ Therefore, the mechanism of formation of ROMPISA nano-objects is not possible to probe by TEM.

Several researchers have investigated the morphological evolution of vesicles formed from small molecule amphiphiles, such as lipids and surfactants, using time-resolved small-angle X-ray scattering (SAXS).⁴²⁻⁴⁴ More recently, this *in situ* technique has been applied to explore the

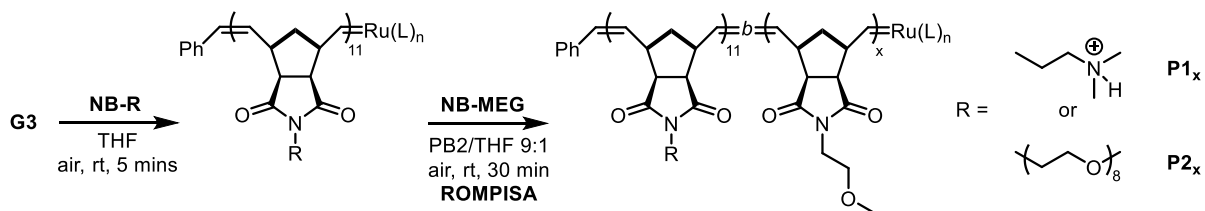
mechanism of formation for block copolymer self-assemblies.⁴⁵⁻⁴⁹ This technique does not require drying or freezing of samples and can provide bulk information.

SAXS is a powerful characterization technique that can provide precise structural information in a non-destructive manner over multiple length scales.⁵⁰ By using high intensity X-rays, acquisition times can be significantly reduced thus allowing a system to be probed in real-time.⁵¹ Due to the rapid polymerisation and self-assembly, *in situ* SAXS analysis is an ideal technique to probe the mechanism of formation of nano-objects formed by ROMPISA.

4.3.3.1 Static SAXS analysis of nano-objects prepared by ROMPISA

ROMPISA nano-objects are prepared at low core monomer concentration (1 wt%) and so, during the early stages of the polymerisation, the concentration of self-assembled structures is very low (< 0.1 wt%). As such, preliminary SAXS experiments in which particles with a core $DP \geq 100$ were targeted did not allow the early stages of nanostructure formation to be probed due to the rapid formation of higher order structures. To obtain reliable data prior to forming polymersomes, and thus “zoom in” on the initial growth of the nanostructures, shorter core DP blocks were targeted as micellation would occur at higher wt%.

As such, amphiphilic block copolymers, **P1_x**, P(**NB-Amine**)₁₁- P(**NB-MEG**)_x, containing a corona formed from **NB-Amine** and a core of **NB-MEG** (DP = 20, 40 or 60) and **P2_x**, P(**NB-PEG**)₁₁- P(**NB-MEG**)_x, containing a corona formed from **NB-PEG** and a core of **NB-MEG** (DP = 20, 40 or 60) were prepared by ROMPISA (Scheme 4.6).



Scheme 4.6. Synthesis of **P1_x** and **P2_x** diblock copolymers by ROMPISA.

SEC analysis of the polymers showed unimodal molecular weight distributions and confirmed successful chain extension of the macroinitiator with low dispersity ($\mathcal{D} \leq 1.3$) for all polymers (Table 4.4 and 4.5 and Figure 4.22).

Table 4.4. Characterisation summary of **P1_x**.

Polymer	$M_{n,\text{theo}}$ / kDa	$M_{n,\text{GPC}}$ / kDa	$\mathcal{D}_{\text{GPC}}$	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}
P(NB-Amine) ₁₁	3.4	2.7	1.18	N/A	N/A
P1₂₀	7.8	7.8	1.14	55	0.55
P1₄₀	12.2	12.8	1.12	23	0.22
P1₆₀	16.7	18.1	1.10	27	0.13

Table 4.5. Characterisation summary of **P2_x**.

Polymer	$M_{n,\text{theo}}$ / kDa	$M_{n,\text{GPC}}$ / kDa	$\mathcal{D}_{\text{GPC}}$	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}
P(NB-PEG) ₁₁	6.6	6.8	1.13	N/A	N/A
P2₂₀	11.0	11.1	1.24	39	0.24
P2₄₀	15.4	14.0	1.22	31	0.22
P2₆₀	19.8	17.8	1.28	32	0.11

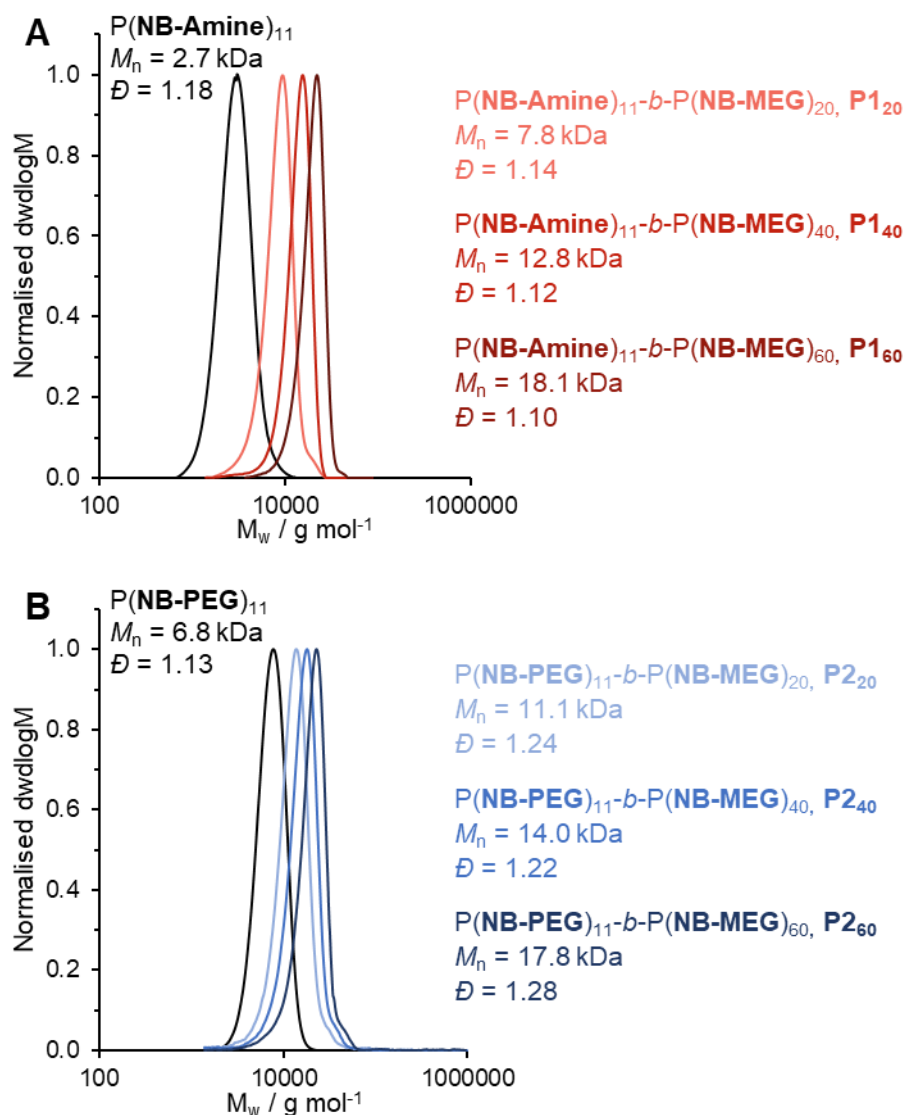


Figure 4.22. Normalised SEC traces (THF eluent, PMMA standards) of (A) **P1_x** and (B) **P2_x**, and intermediate polymers.

The resultant solutions were analysed by DLS which showed unimodal distributions for **P1₆₀** and **P2₆₀** (Table 4.4 and 4.5 Figure 4.23). Polymers with core DP < 60 displayed small secondary peaks, which can be attributed to a small amount of aggregation. DLS analysis of **P1₂₀** showed a multimodal distribution with a flatter correlation curve, indicating the presence of either a mixed morphology or unstable nanostructures in solution.

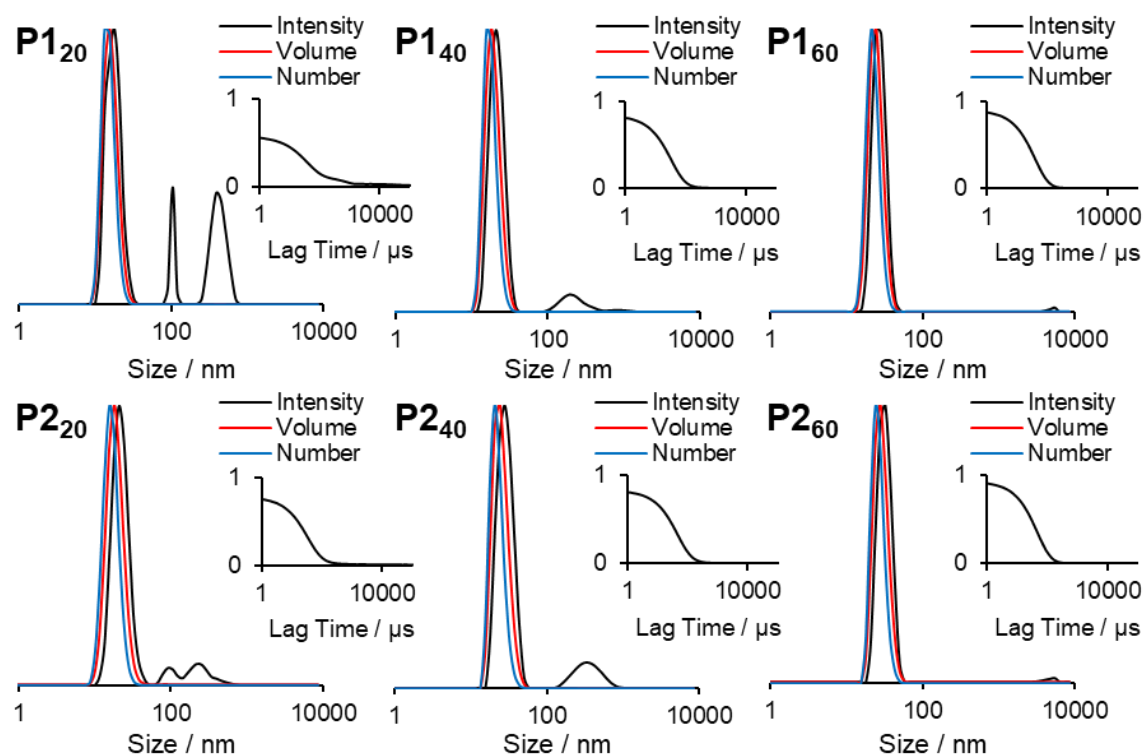


Figure 4.23. Normalised DLS analysis of **P1_x** and **P2_x**.

In addition, static SAXS analysis on preformed particles was also conducted (Figure 4.24). As for previous SAXS analysis of polymersomes obtained by ROMPISA, data obtained for **P1_x** and **P2_x** were best fitted using a spherical micelle model⁵² (i.e. with a solid polymer core) rather than a vesicle model (Figure 4.24, A and B).⁵³ This is due to the thick membrane being of the order of the overall particle radius, and thus, the model is insensitive to such a small lumen.²⁸

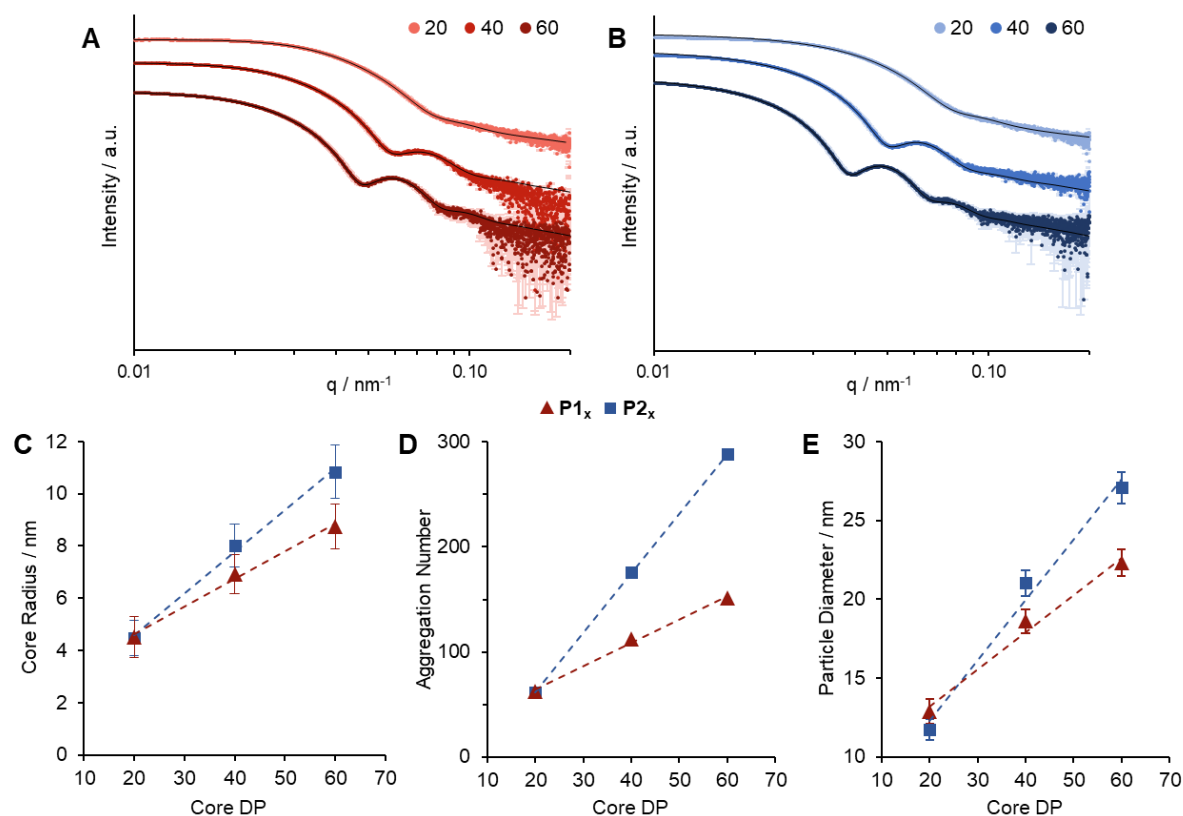


Figure 4.24. Staggered SAXS patterns of (A) $P1_x$ and (B) $P2_x$ fitted to a spherical micelle model (black line). Core DP dependent trends in (C) core radius, (D) aggregation number and (E) particle diameter for $P1_x$ (red) and $P2_x$ (blue) obtained by SAXS analysis.

Size data extracted from modelling the SAXS patterns of $P1_x$ and $P2_x$ show a clear trend of increasing core radius, aggregation number and particle diameter as core DP was increased (Figure 4.24). This is in agreement with the DLS data, as well as in a previous ROMPISA report.⁸

4.3.3.2 In situ SAXS analysis of nano-objects prepared by ROMPISA

Following this, the formation of $P1_x$ and $P2_x$ nano-objects was studied by time-resolved SAXS analysis using a stopped-flow apparatus. Pre-formed corona blocks of P(NB-Amine) ($P1_x$) or P(NB-PEG) ($P2_x$) were rapidly mixed with a solution of NB-MEG dissolved in 100 mM PB2 and flowed into the measurement capillary within 10 ms.

Scattering data from the resultant solution was acquired during the 30 min reaction time in three collection programs: (1) 1 s intervals (500 ms exposure) for 10 mins; (2) 5 s intervals (1 s exposure) for 10 mins; (3) and 10 s intervals (1 s exposure) for 10 mins (Figure 4.25, S4.8 and S4.9).

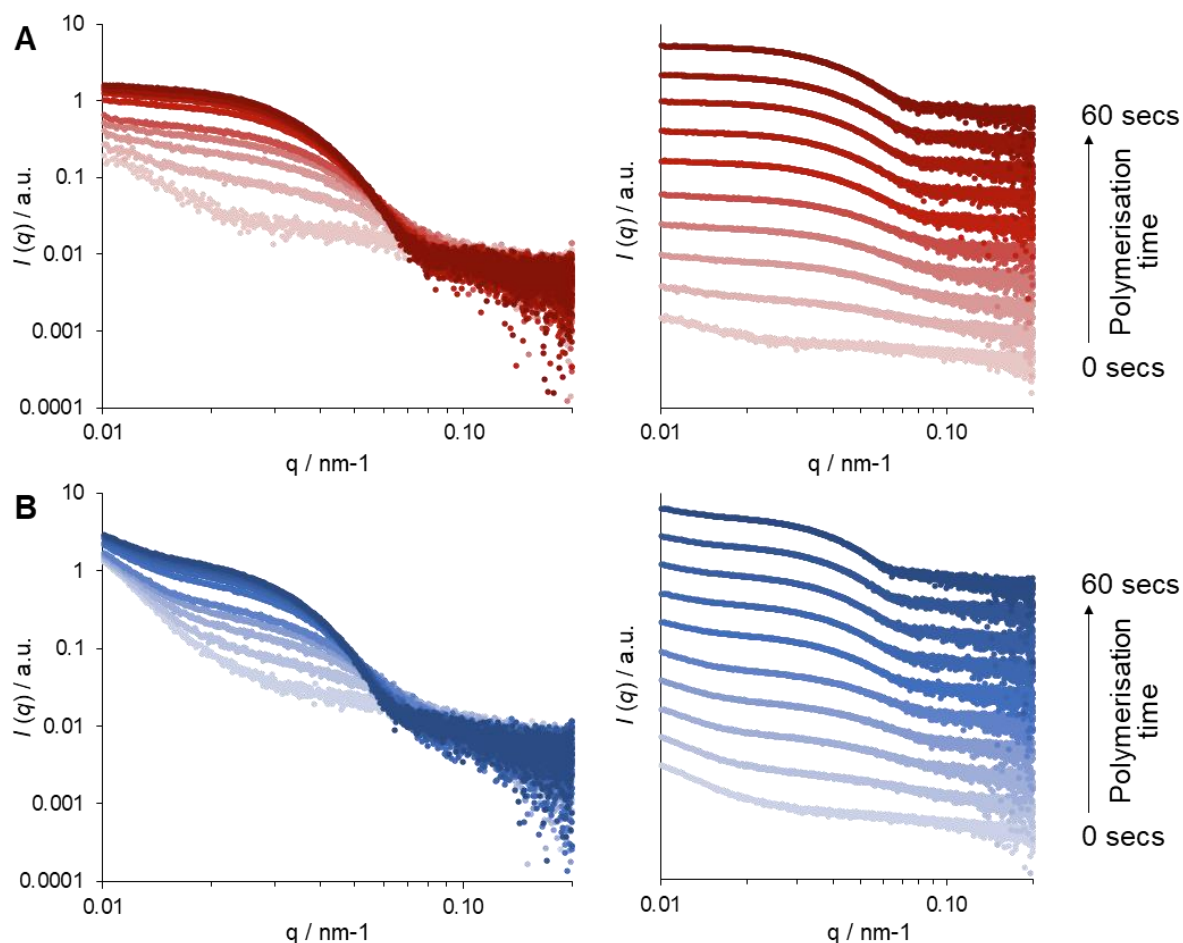


Figure 4.25. Overlaid (left) and staggered (right) SAXS patterns for (A) **P1₄₀** and (B) **P2₄₀**.

No significant change in scattering was observed after 10 minutes for all ROMPISA formulations. As such, data obtained after 10 minutes was omitted for clarity. *In situ* SAXS analysis indicated the presence of nano-objects as early as 5 seconds, providing further evidence of the rapid polymerisation and self-assembly of ROMPISA highlighted in other reports.^{28, 33, 54}

The SAXS patterns taken at 10 minutes for **P1₂₀** and **P2₂₀** nano-objects showed a significant shift of the inflexion point, indicating an increased particle size when compared to the static patterns of the same nano-object formulation formed by conventional laboratory experiments (Figure 4.26). The disparities may be due to these formulations being close to the critical DP for self-assembly, resulting in poor colloidal stability of the nano-objects. This was also evidenced by DLS analysis.

Endpoint SAXS patterns for **P1_x** and **P2_x** nano-objects above core DP 20 were better matched with the static patterns of nano-objects formed by conventional laboratory experiments, although a smoothing of the *in situ* curves indicates a greater dispersity of nano-object size throughout the sample. (Figure 4.26). The differences between the experimental set ups (e.g. pipette mixing vs. flow mixing, exposure to X-rays, etc.) may have affect the polymerisation rate and/or self-assembly kinetics, resulting in a different final nano-object structure. Only analysis of nano-objects with core DP > 20 will be discussed from this point.

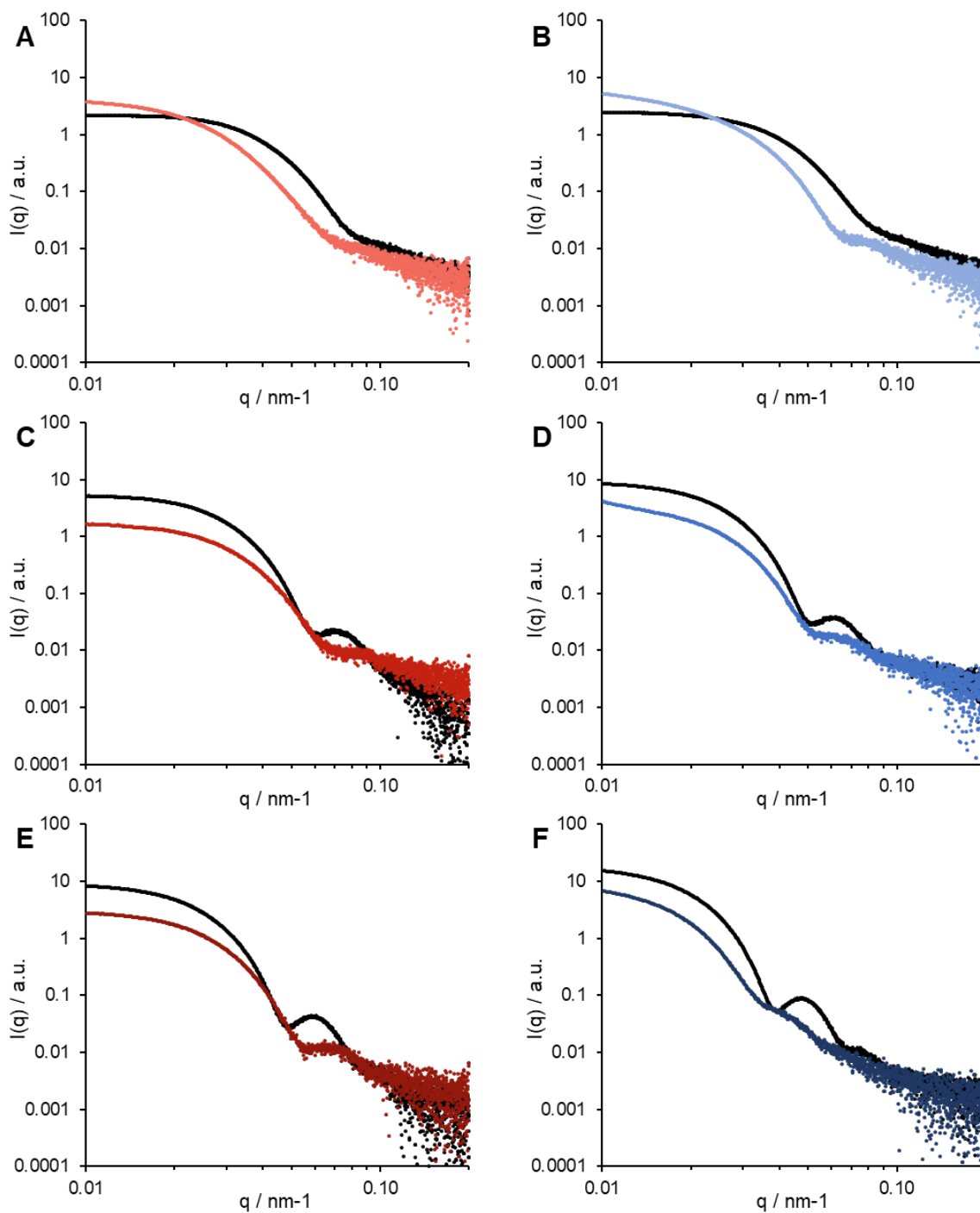


Figure 4.26. Overlaid SAXS patterns of static (black) and *in situ* endpoints (red or blue) measurements for (A) **P1₂₀**, (B) **P2₂₀**, (C) **P1₄₀**, (D) **P2₄₀**, (E) **P1₆₀** and (F) **P2₆₀**.

Time-resolved SAXS data between 1 and 600 seconds were fitted to the spherical model previously employed for the static data (Figure 4.27). Satisfactory fits were obtained for all

SAXS patterns using this approach with an increase in core radius, aggregation number and particle size extracted over the duration of the experiment.

For the **P1_x** particles, the core radius of the particles increased quickly over the first 40 seconds before plateauing for all three samples, which may suggest the ROMPISA systems are rapidly forming polymersomes, of which the membranes then grow inwards as the reaction proceeds. This result is also observed in **P2₄₀**, whereas the core radius in **P2₆₀** continued to increase until 8 minutes. This rapid growth and subsequent plateau were also observed in the aggregation number and overall particle size of **P1_x** and **P2₄₀** samples, with **P2₆₀** again showing a lengthened growth period.

It was not possible to adequately fit a cylindrical micelle model to any pattern, indicating no anisotropic particles formed during the reaction. Mechanism I proceeds *via* a transition from isotropic micelles to anisotropic bilayer layers, before returning to isotropic vesicles following closure. The absence of divergence from an isotropic spherical model throughout the timescale of the reaction provides tentative evidence that ROMPISA nano-objects form *via* mechanism II. However, due to the requirement to fit the final morphology to a spherical micelle model, further analysis is required to confirm the presence of polymersomes and the mechanism of their formation.

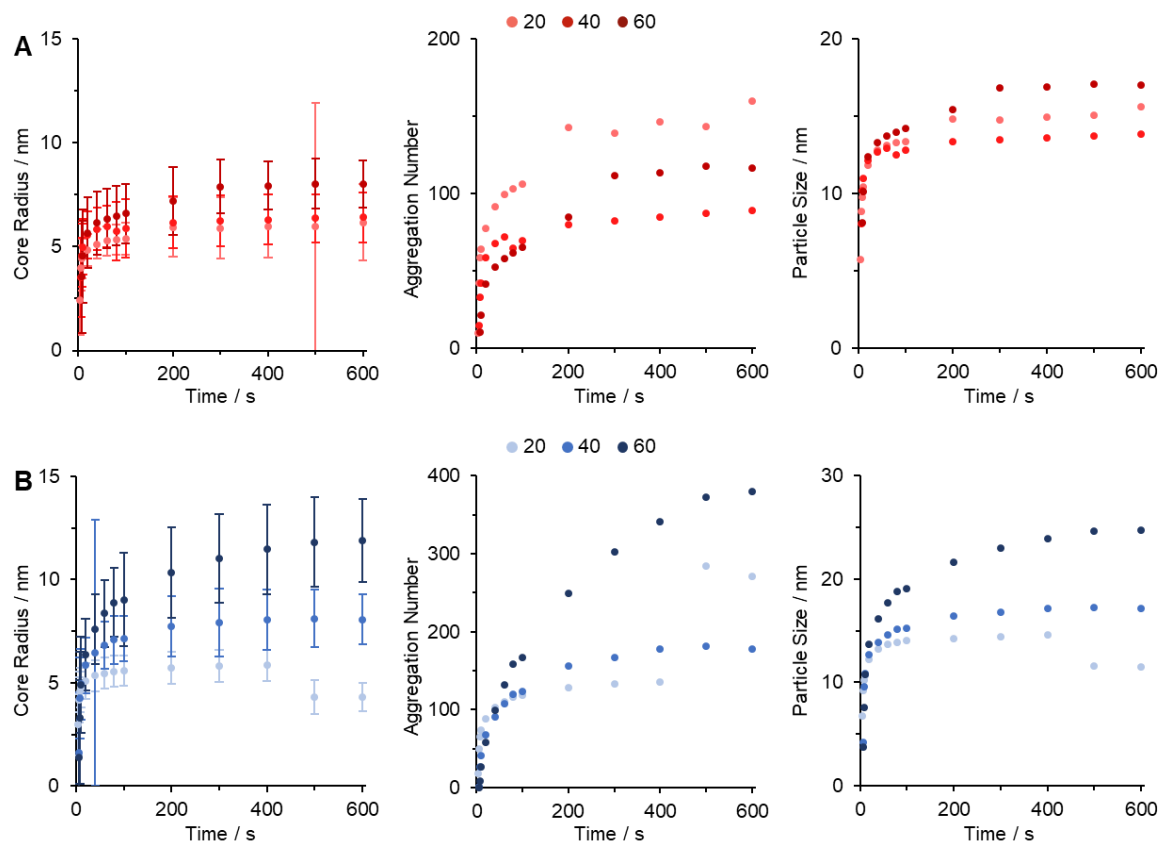


Figure 4.27. Evolution of core radius, aggregation number and particle size as a function of time for (A) **P1_x** and (B) **P2_x** nano-objects, as determined by modelling scattering patterns obtained by *in situ* SAXS analysis. Error bars indicate the standard deviation of the core radius.

4.4 Conclusion

In this work, the encapsulation ability of diblock copolymer nano-objects prepared *via* ROMPISA was investigated. HPTS was shown to disrupt the self-assembly process for nano-objects with amine-functionalised corona blocks due to the shielding of the positive charge, but had little impact on the formation of uncharged, PEG-functionalised nano-objects.

Purification of dye from nano-objects was shown to be pH dependent, with dialysis at pH 7 resulting in an increase in fluorescence. In contrast, fluorescence spectroscopy following pH 2 dialysis purification indicated an encapsulation efficiency of 0.23% for the ROMPISA nano-objects, lower than previous reports for PISA encapsulation. Gel permeation chromatography was tested as an alternative purification method as it had a shorter timescale and would reduce the impact of any dye leakage that may have occurred during dialysis. However, the resulting solutions showed no improvement in encapsulation.

The impact of inorganic NP encapsulation on ROMPISA self-assembly was probed. Silica NPs were shown to impact both the polymerisation and self-assembly of the nano-objects which was attributed to non-covalent interactions between the PEG corona and the silica. In contrast, gold NPs did not have an impact on ROMPISA self-assembly but were shown to aggregate to the outside of the nano-objects rather than be encapsulated within the internal lumen. It was therefore concluded that water-soluble cargo was unable to be encapsulated during ROMPISA self-assembly.

Following this, the mechanism of formation of ROMPISA was probed by SAXS. Static measurements demonstrated an increase in core radius, aggregation number and particle diameter as core DP is increased. Time-resolved SAXS analysis indicated the presence of nano-

objects as early as 5 seconds, with patterns for nano-objects above core DP 20 showing good agreement between static measurements of pre-formed particles and the *in situ* endpoints. All time points were modelled to a spherical micelle model, indicating no anisotropic particles were formed during ROMPISA.

These results indicate ROMPISA nano-objects form by mechanism II, for which encapsulation is precluded during nano-object self-assembly. These results further the understanding of the self-assembly process during ROMPISA and highlight the importance of the mechanism by which these structures form when assessing their efficacy for potential applications.

4.5 Experimental

4.5.1 Materials

Unless otherwise stated reagents were obtained from commercial sources and used without purification. Tetrahydrofuran (THF) (HPLC grade) was purchased from VWR Chemicals and was purified *via* passage through a column of neutral alumina prior to use. Formvar-carbon coated (300 mesh) copper grids were purchased from EM Resolutions. Dialysis membranes were purchased from Fisher Scientific (Thermo Scientific™ SnakeSkin™, 3.5 kDa MWCO, cellulose, 22 mm I.D.). Spin filters were purchased from MilliporeSigma (Amicon® Ultra centrifugal filters, 100 kDa). Gel filtration columns were purchased from Cytiva (PD-10 desalting columns packed with Sephadex G-25 resin). Colloidal silica solution was purchased from Sigma-Aldrich (Ludox® AM, 30 wt%). Gold nanoparticles were purchased from Cytodiagnostics (5 nm core size, citrate stabilised, 91 nM in 0.1 mM phosphate buffered saline).

G3 (modified Grubbs 3rd generation catalyst, $((\text{H}_2\text{IMes})(\text{pyr})_2(\text{Cl})_2\text{Ru}=\text{CHPh}))$), **NB-PEG**, **NB-Amine** and **NB-MEG** were prepared as previously described.^{54, 55, 56}

4.5.2 Characterisation Techniques

4.5.2.1 Size Exclusion Chromatography

Size exclusion chromatography (SEC) analysis was performed on a system composed of an Agilent 1260 Infinity II LC system equipped with an Agilent guard column (PLGel 5 μM , 50 $\times$ 7.5 mm) and two Agilent Mixed-C columns (PLGel 5 μM , 300 $\times$ 7.5 mm). The mobile phase used was THF (HPLC grade) containing 2% v/v NEt_3 at 40 °C with a flow rate of 1.0 mL min⁻¹ (poly(methyl methacrylate) (PMMA) standards used for calibration). Number average

molecular weight (M_n), weight average molecular weight (M_w) and dispersity ($\bar{D} = M_w/M_n$) were determined using Agilent GPC/SEC software.

4.5.2.2 Dynamic Light Scattering

Hydrodynamic diameters (D_h) and size distributions (PD) of nano-objects were determined by dynamic light scattering (DLS) and microelectrophoresis using a Malvern Zetasizer Nano ZS with a 4 mW He-Ne 633 nm laser module operating at 25 °C. Samples were diluted to give a final polymer concentration of 0.01 wt%. Measurements were carried out at an angle of 173° (back scattering), and results were analysed using Malvern DTS v7.03 software. All determinations were repeated five times with at least 10 measurements recorded for each run. D_h values were calculated using the Stokes-Einstein equation where particles are assumed to be spherical, while for anisotropic particles DLS was used to indicate a change in average particle size and obtain dispersity information.

4.5.2.3 Transmission Electron Microscopy

Dry-state, stained transmission electron microscopy (TEM) imaging was performed on a JEOL JEM-1400 microscope operating at an acceleration voltage of 80 kV. All dry-state samples were diluted to an appropriate analysis concentration and then deposited onto formvar-coated grids. After roughly 1 min, excess sample was blotted from the grid and the grid was then stained with an aqueous 1 wt% uranyl acetate (UA) solution for 1 min prior to blotting, drying and microscopic analysis. Images were analysed using ImageJ software in which a minimum of 100 particles were measured.

4.5.2.4 Fluorescence Spectroscopy

Fluorescence emission and excitation spectra were obtained using an Edinburgh Instruments FS5 Spectrofluorometer in quartz 3.5 mL cuvettes for liquid samples (Starna Cell, Type: 3/Q/10), and analysed in Fluoracle (Edinburgh Instruments).

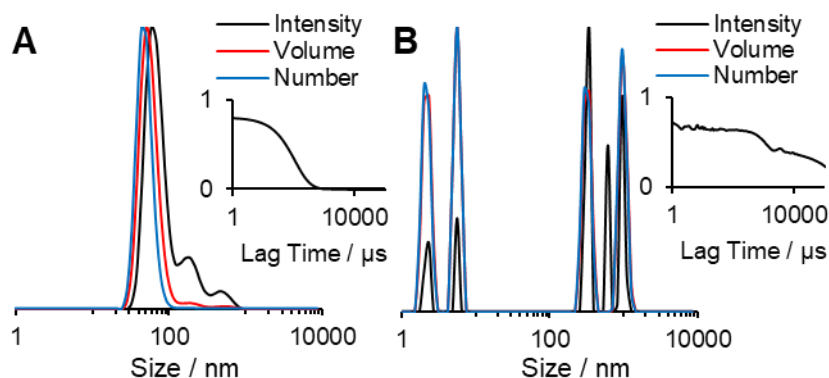
4.5.2.5 Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) patterns were recorded at a synchrotron source (Diamond Light Source, station I22, Didcot, UK; Experiment ID SM33098) using monochromatic X-ray radiation (X-ray wavelength $\lambda = 1.00 \text{ \AA}$, with scattering vector q ranging from 0.0017 to $0.17 \text{ \AA}^{-1}$, where $q = 4\pi \sin \theta / \lambda$ and θ is one-half of the scattering angle) and a 2D Pilatus 2M pixel detector (Dectris, Switzerland). All static SAXS measurements were performed on 1.0% w/w copolymer dispersions in 2.0 mm glass capillaries. Time-resolved experiments were performed using a BioLogic SFM-400 stopped-flow mixing system equipped with an umbilical connector, mixing unit and observation cell containing a 1.0 mm glass capillary, without delay lines: a solution of either P(**NB-Amine**)₁₁ or P(**NB-PEG**)₁₁ macroinitiator ($4.4 \text{ } \mu\text{mol mL}^{-1}$ for DP 20, $2.2 \text{ } \mu\text{mol mL}^{-1}$ for DP 40, and $1.5 \text{ } \mu\text{mol mL}^{-1}$ for DP 60) in THF/PB2 (2:9 v/v) and a 10 mg mL^{-1} solution of **NB-MEG** in PB2 were loaded into two separate 10 mL syringes before $400 \text{ } \mu\text{L}$ of each solution was flowed into the observation cell at 3.33 mL s^{-1} so that a final copolymer concentration of 1% w/w and a final solvent composition of THF/PB2 of 1:9 v/v was obtained on mixing. Scattering data were reduced and normalised, with glassy carbon being used for the absolute intensity calibration, utilizing standard routines available at the beamline⁵⁷ and further analysed (background subtraction and data modelling) using Irena SAS macros for Igor Pro.⁵⁸

4.5.3 Encapsulation of HPTS within ROMPISA nano-objects

4.5.3.1 Synthesis of nano-objects *via* ROMPISA

P1₁₂₀ and **P2₁₂₀** nano-objects were synthesised according to reference 33.



4.5.4 Purification of HPTS from nano-objects *via* ROMPISA

4.5.4.1 Dialysis

ROMPISA solutions (pH = 2, PB2, final solids concentration = 1 wt%) were diluted x100 in PB2 buffer and transferred to a dialysis bag made from SnakeSkin dialysis tubing (3.5 kDa MWCO). The sample was placed in a beaker containing 500 mL of external dialysis solution (H₂O or HCl solution) and stirred slowly at room temperature for 3 days. The exterior solution was replaced 3 times over the 3-day period.

Table S4.1. Characterisation summary of **P2₁₂₀** after dialysis at pH 2.

Polymer	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
P2₁₂₀ , after dialysis at pH 2	55	0.33	69 ± 5

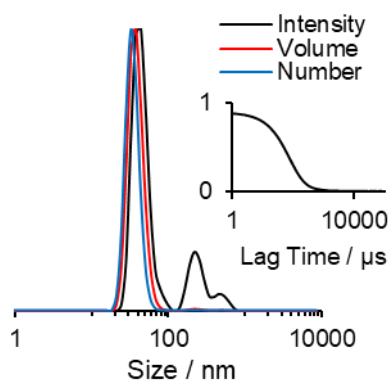


Figure S4.2. Normalised DLS analysis of **P2₁₂₀** after dialysis at pH 2.

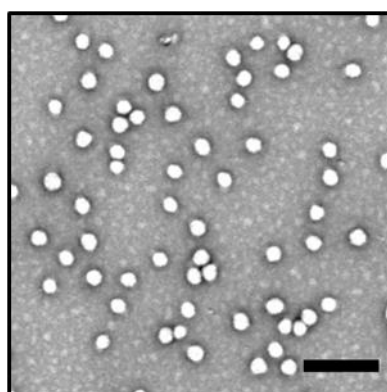


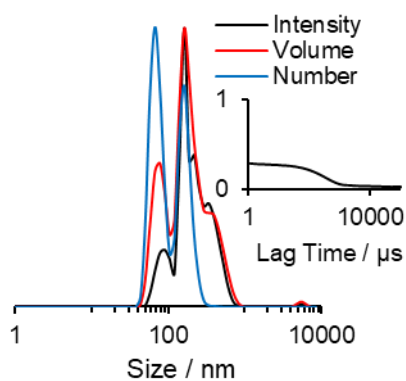
Figure S4.3. Dry-state TEM image of **P2₁₂₀** nano-objects after dialysis at pH 2. Scale bar = 200 nm.

4.5.4.2 Spin Filtration

ROMPISA solutions (pH = 2, PB2, final solids concentration = 1 wt%) were diluted x100 in PB2 buffer and transferred to a spin filter (100 kDa). The filter was centrifuged for 10 mins at 14,000 x g (19°C). The supernatant was removed and fresh PB2 buffer added to the spin filter. This process was repeated three times in total. Following this, the spin filter was inverted in the tube and centrifuged for 4 mins at 1000 x g (19°C). PB2 buffer was added give 500 μL in total.

Table S4.2. Characterisation summary of **P2₁₂₀** after spin filtration.

Polymer	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}
P2₁₂₀ , after spin filtration	250	0.36

**Figure S4.4.** Normalised DLS analysis of **P2₁₂₀** after purification by spin filtration.

4.5.4.3 Gel Filtration Chromatography

Gel filtration columns were first equilibrated. The storage solution was removed from the top of the column and the sealed end cut to form an opening. 4.5 mL of PB2 buffer was added to the top of the tube and allowed to fully enter the packed bed. This was repeated 4 times and the flow-through discarded each time.

ROMPISA solutions (pH = 2, PB2, final solids concentration = 1 wt%) were diluted x100 in PB2 buffer and added to the top of the column. The solution was allowed to enter the packed bed completely and the flow-through discarded. The sample was then eluted with 6 mL of PB2 buffer and collected in a glass vial.

Table S4.3. Characterisation summary of **P2₁₂₀** after gel filtration chromatography.

Polymer	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
P2₁₂₀ , after gel filtration chromatography	62	0.22	43 ± 3

4.5.5 Concentration dependence of HPTS

4.5.5.1 Synthesis of nano-objects *via* ROMPISA

Table S4.4. Fluorescence analysis of **P2₁₂₀** synthesised with different concentrations of HPTS before and after dialysis.

[HPTS] / mg mL ⁻¹	Normalised fluorescence emission intensity ($\lambda_{\text{Em}} = 512 \text{ nm}$) before dialysis	Normalised fluorescence emission intensity ($\lambda_{\text{Em}} = 512 \text{ nm}$) after dialysis
0	0.02	1.2 x 10 ⁻³
1	0.19	0.9 x 10 ⁻³
10	0.66	1.3 x 10 ⁻³
100	1.00	2.8 x 10 ⁻³

4.5.6 Encapsulation of inorganic NPs within ROMPISA nano-objects

4.5.6.1 Synthesis of nano-objects *via* ROMPISA

P3₁₈₀ nano-objects were synthesised analogously to reference 33.

Table S4.5. Characterisation summary of **P3₁₈₀**.

Polymer	$M_{n,\text{theo}}$ / kDa	$M_{n,\text{GPC}}$ / kDa	$\bar{D}_{\text{GPC}}$	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
P(NB-PEG) ₁₅	8.7	10.1	1.16	N/A	N/A	N/A
P3₁₈₀	48.5	40.3	1.23	106	0.46	64 ± 6

4.5.6.2 Silica NPs

Table S4.6. Characterisation summary of silica NPs.

Sample	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
Silica NPs (1 wt %)	30	0.20	19 ± 3

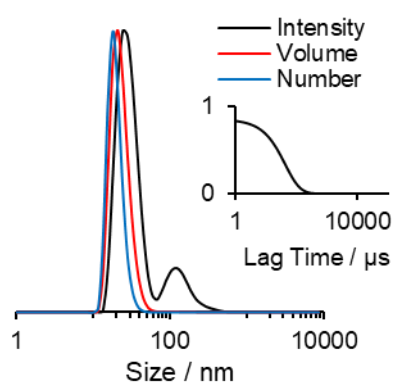
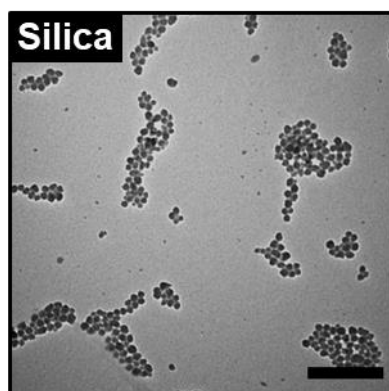
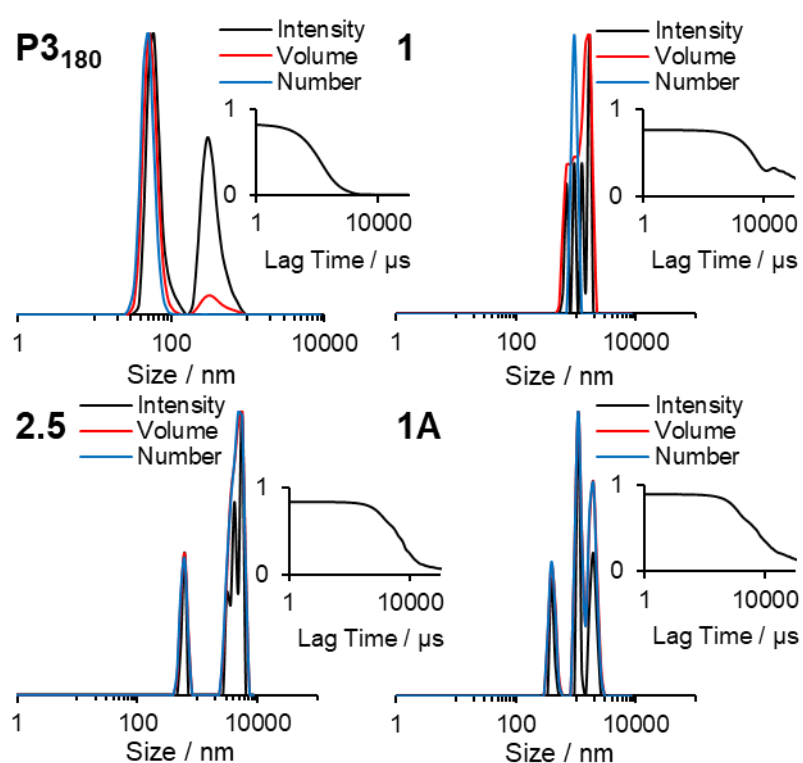
**Figure S4.5.** Normalised DLS analysis of silica NPs (1 wt %).**Figure S4.6.** Dry-state TEM image of silica NPs (1 wt %). Scale bar = 200 nm.

Table S4.7. Characterisation summary of **P3₁₈₀** synthesised with silica NPs.

[Silica NP] / wt %	$M_{n,theo}$ / kDa	$M_{n,GPC}$ / kDa	$\bar{D}_{GPC}$	$Z_{avg,DLS}$ / nm	PDI_{DLS}
1	48.5	51.8	1.45	5138	0.82
2.5	N/A	N/A	N/A	4319	0.31
1A (added after)	N/A	N/A	N/A	3832	0.54

**Figure S4.7.** Normalised DLS analysis of native **P3₁₈₀** particles; **P3₁₈₀** particles synthesised in the presence of 1 wt% silica (1), 2 wt% silica (2), and without silica present but added after self-assembly (1A).

4.5.6.2 Gold NPs

Table S4.8. Characterisation summary of gold NPs.

Sample	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
Gold NPs (OD = 0.5)	18	0.49	4 ± 0

Table S4.9. Characterisation summary of **P3₁₈₀** synthesised with gold NPs.

[Gold NP] / OD	$Z_{\text{avg,DLS}}$ / nm	PDI_{DLS}	L_{TEM} / nm
0.01	77	0.45	N/A
0.1	61	0.35	N/A
0.25	94	0.50	N/A
0.5	110	0.59	67 ± 8

4.5.7 Mechanism of Formation

4.5.7.1 SAXS modelling

Programming tools within the Irena SAS macros for Igor Pro were used to model experimental SAXS data.⁵⁹ Models used to fit SAXS data in this work were previously developed by Pedersen.⁵²

In general, the intensity of X-rays scattered by a dispersion of nano-objects [as represented by the scattering cross-section per unit sample volume, $\frac{d\Sigma}{d\Omega}(q)$] can be expressed as:

$$\frac{d\Sigma}{d\Omega}(q) = NS(q) \int_0^\infty \dots \int_0^\infty F(q, r_1, \dots, r_k)^2 \Psi(r_1, \dots, r_k) dr_1, \dots, dr_k \quad \text{S4.1}$$

where $F(q, r_1, \dots, r_k)$ is the form factor, $r_1, \dots, r_k$ is a set of k parameters describing the nano-object structural morphology, $\Psi(r_1, \dots, r_k)$ is the distribution function, $S(q)$ is the structure factor and N is the number density of nano-objects per unit volume expressed as:

$$N = \frac{\varphi}{\int_0^\infty \dots \int_0^\infty V(r_1, \dots, r_k) \Psi(r_1, \dots, r_k) dr_1, \dots, dr_k} \quad \text{S4.2}$$

where $V(r_1, \dots, r_k)$ is the nano-object volume and φ is the volume fraction of the nano-objects within the dispersion. It is assumed that $S(q) = 1$ at the sufficiently low copolymer concentrations used in this study ($\leq 1.0\%$ w/w).

For this study, experimental SAXS data required fitting to the spherical micelle model. Additionally, optimum fits often required the use a power law relationship (herein described as a unified fit) to adequately fit low- q data, respectively. Thus, the intensity of scattering at a given q vector, $I(q)$, is expressed as:

$$I(q) = \frac{d\Sigma}{d\Omega}(q)_{sphere} + Bq^{-P} \quad \text{S4.3}$$

where $\frac{d\Sigma}{d\Omega}(q)_{sphere}$ is the form factor for spherical micelles, and terms B and P are constants.

Spherical micelle model

The spherical micelle form factor for Equation S1, which contributes to $\frac{d\Sigma}{d\Omega}(q)_{sphere}$ is given by:

$$\begin{aligned} F_{smic}(q) = & N_s^2 \beta_s^2 A_s^2(q, R_s) + N_s \beta_c^2 F_c(q, R_g) + N_s(N_s - 1) \beta_c^2 A_c^2(q) \\ & + 2N_s^2 \beta_s \beta_c A_s(q, R_s) A_c(q) \end{aligned} \quad \text{S4.4}$$

where R_s is the volume-average sphere core radius and R_g is the radius of gyration of the coronal steric stabilizer block. The X-ray scattering length contrasts for the core and corona blocks are given by $\beta_s = V_s(\xi_s - \xi_{sol})$ and $\beta_c = V_c(\xi_c - \xi_{sol})$, respectively. Here, ξ_s , ξ_c and ξ_{sol} are the X-ray scattering length densities of the core block ($\xi_{P(NB-MEG)} = 10.77 \times 10^{10} \text{ cm}^{-2}$), corona block ($\xi_{P(NB-PEG)} = 10.31 \times 10^{10} \text{ cm}^{-2}$ or $\xi_{P(NB-Amine)} = 11.59 \times 10^{10} \text{ cm}^{-2}$) and water ($\xi_{sol} = 9.42 \times 10^{10} \text{ cm}^{-2}$), respectively. V_s and V_c are the volumes of the core block and the corona block, respectively. Values for V_s and V_c were calculated using $V = \frac{M_{n_{pol}}}{N_A \rho}$ taking the solid-state homopolymer densities of P(NB-MEG) and P(NB-Amine) determined by helium pycnometry ($\rho_{P(NB-MEG)} = 1.19 \text{ g cm}^{-3}$ and $\rho_{P(NB-Amine)} = 1.27 \text{ g cm}^{-3}$) and P(NB-PEG), which was taken to equal that of PEG ($\rho_{P(NB-PEG)} = 1.13 \text{ g cm}^{-3}$),⁶⁰ where $M_{n_{pol}}$ is the number-average molecular weight of each polymer block determined by ¹H NMR spectroscopy. The sphere form factor amplitude is used for the amplitude of the core self-term:

$$A_c(q, R_s) = \Phi(qR_s) \exp\left(-\frac{q^2 \sigma^2}{2}\right) \quad \text{S4.5}$$

where $\Phi(qR_s) = \frac{3[\sin(qR_s) - qR_s \cos(qR_s)]}{(qR_s)^3}$. A sigmoidal interface between the two blocks was assumed for the spherical micelle form factor (Equation S4). This is described by the exponent term with a width σ accounting for a decaying scattering length density at the micellar interface. This σ value was fixed at 2.2 during fitting.

The form factor amplitude of the spherical micelle corona is:

$$A_c(q) = \frac{\int_{R_s}^{R_s+2s} \mu_c(r) \frac{\sin(qr)}{qr} r^2 dr}{\int_{R_s}^{R_s+2s} \mu_c(r) r^2 dr} \exp\left(-\frac{q^2 \sigma^2}{2}\right) \quad \text{S4.6}$$

The radial profile, $\mu_c(r)$, can be expressed by a linear combination of two cubic b splines, with two fitting parameters s and a corresponding to the width of the profile and the weight coefficient, respectively. This information can be found elsewhere,⁶² as can the approximate integrated form of Equation S5. The self-correlation term for the coronal block is given by the Debye function:

$$F_c(q, R_g) = \frac{2[\exp(-q^2 R_g^2) - 1 + q^2 R_g^2]}{q^4 R_g^4} \quad \text{S4.7}$$

where R_g is the radius of gyration of the coronal block. The aggregation number, N_s , of the spherical micelle is given by:

$$N_s = (1 - x_{sol}) \frac{\frac{4}{3}\pi R_s^3}{V_s} \quad \text{S4.8}$$

where x_{sol} is the volume fraction of solvent within the P(**NB-MEG**) micelle cores, which was found to be zero in all cases. A polydispersity for one parameter (R_s) is assumed for the micelle model, which is described by a Gaussian distribution. Thus, the polydispersity function in Equation S1 can be represented as:

$$\psi(r_1) = \frac{1}{\sqrt{2\pi\sigma_{R_s}^2}} \exp\left(-\frac{(r_1 - R_s)^2}{2\sigma_{R_s}^2}\right) \quad \text{S4.9}$$

where σ_{R_s} is the standard deviation for R_s . In accordance with Equation S2, the number density per unit volume for the micelle model is expressed as:

$$N = \frac{\varphi_s}{\int_0^\infty V(r_1) \psi(r_1) dr_1} \quad \text{S4.10}$$

where φ_s is the total volume fraction of copolymer in the spherical micelles and $V(r_1)$ is the total volume of copolymer within a spherical micelle [$V(r_1) = (V_s + V_c)N_s(r_1)$].

4.5.7.2 In situ SAXS analysis

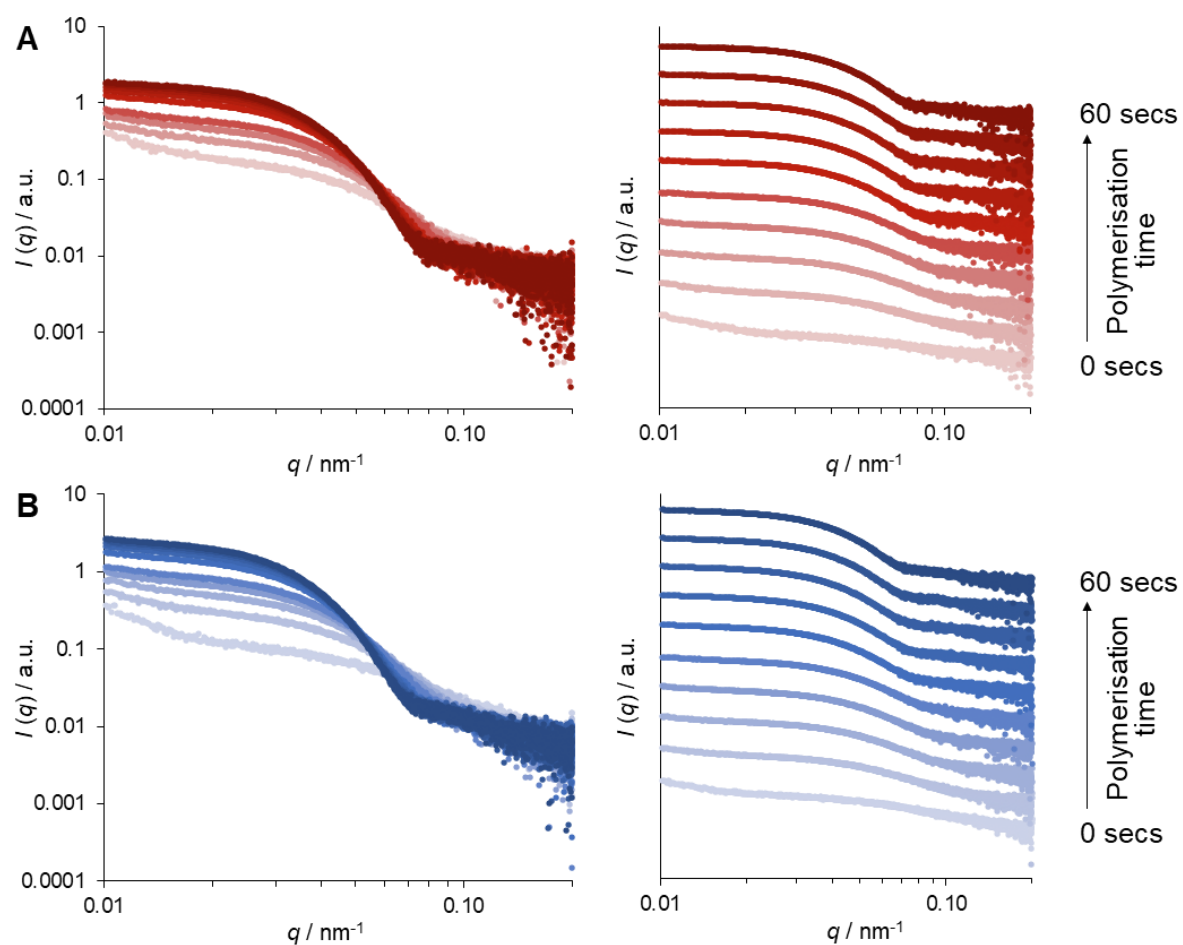


Figure S4.8 Overlaid (left) and staggered (right) SAXS patterns for (A) **P1₂₀** and (B) **P2₂₀**.

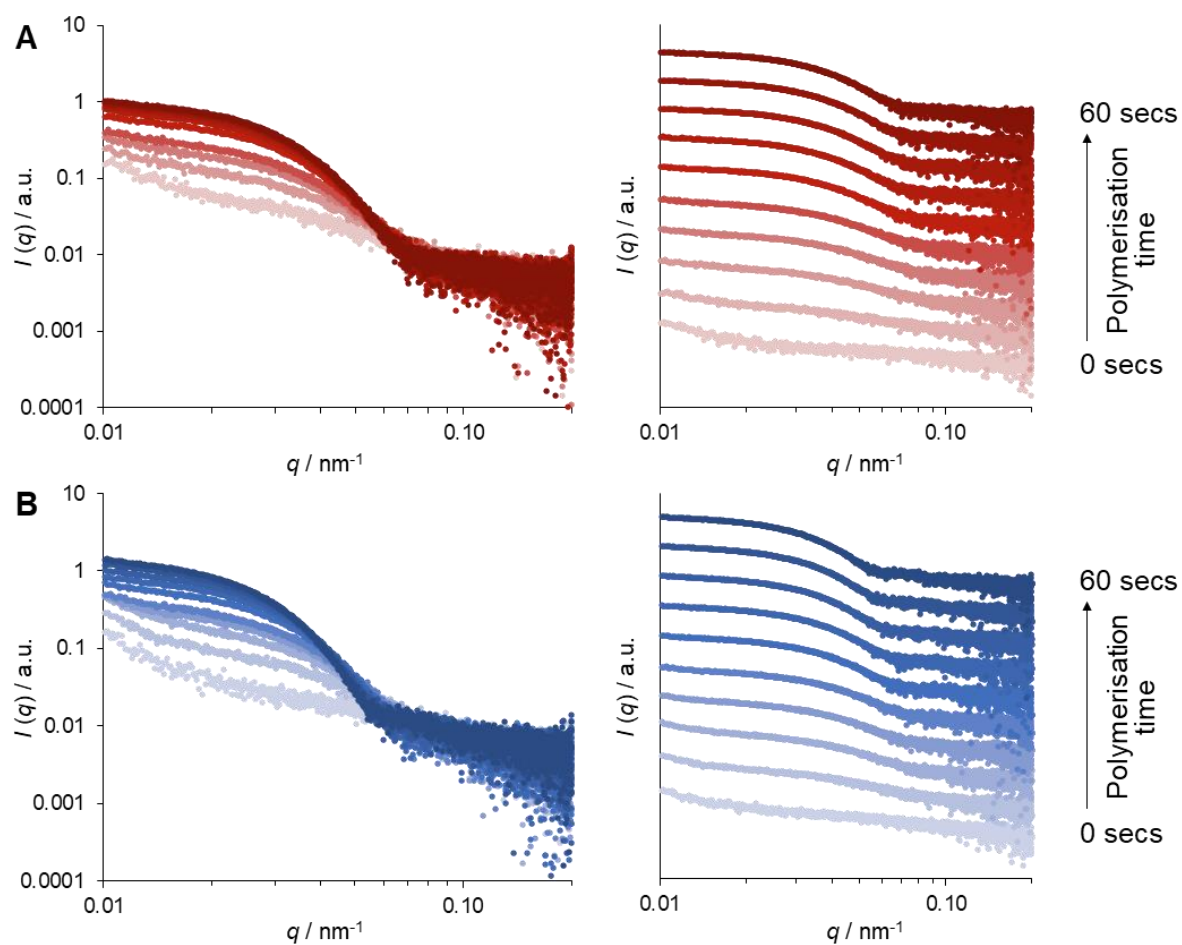


Figure S4.9 Overlaid (left) and staggered (right) SAXS patterns for (A) **P1₆₀** and (B) **P2₆₀**.

Table S4.10. Summary of variable fitting parameters for **P1₂₀** SAXS data.

	Sample	Spherical micelle model			Power law	
		φ_s	R_s (Å)	σ_{R_s} (Å)	B	P
Static		0.0305	45.2	7.87	0	0
Time-resolved	2 s	Unsatisfactory fit				
	4 s	0.00601	24.2	16.6	0.00000219	2.69
	6 s	0.00492	39.7	10.8	0.00000137	2.78
	8 s	0.00507	44.2	9.54	0.00000121	2.83
	10 s	0.00623	45.5	8.89	0.00000131	2.73
	20 s	0.00933	48.5	7.72	0.000000373	3.00
	40 s	0.0115	51.3	7.18	0.0000000819	3.28
	60 s	0.0119	52.7	7.15	0.0000000525	3.33
	80 s	0.0120	53.4	7.32	0.0000000566	3.33
	100 s	0.0119	53.8	7.52	0.0000000820	3.26
	200 s	0.0110	59.5	14.2	0.0000000929	3.25
	300 s	0.0108	58.9	14.8	0.000000142	3.18
	400 s	0.0107	59.9	15.2	0.000000276	3.06
	500 s	0.0114	59.6	59.6	0.000000350	3.00
	600 s	0.0109	61.8	18.4	0.000000281	3.04

Table S4.11. Summary of values calculated using SAXS fitting parameters outlined in Table S4.10 for P1₂₀.

Sample		Mean sphere diameter, D_s (nm) $D_s = 2R_s + 4R_g$	Mean aggregation number, N_{agg} $N_{agg} = \frac{\frac{4}{3}\pi R_s^3}{V_s}$
Static		12.9	62.5
Time-resolved	2 s	Unsatisfactory fit	
	4 s	5.75	9.56
	6 s	8.85	42.3
	8 s	9.76	58.7
	10 s	10.4	64.1
	20 s	11.8	77.2
	40 s	12.8	91.4
	60 s	13.1	99.3
	80 s	13.3	103
	100 s	13.3	106
	200 s	14.8	143
	300 s	14.7	139
	400 s	15.0	146
	500 s	15.1	143
	600 s	15.6	160

Table S4.12. Summary of variable fitting parameters for **P1₄₀** SAXS data.

	Sample	Spherical micelle model			Power law	
		φ_s	R_s (Å)	σ_{R_s} (Å)	B	P
Static		0.0308	69.1	7.47	0	0
Time-resolved	2 s	Unsatisfactory fit				
	4 s	Unsatisfactory fit				
	6 s	0.00585	35.3	19.0	0.00000163	2.70
	8 s	0.00532	45.8	15.1	0.000000194	3.10
	10 s	0.00540	49.9	13.4	0.0000000350	3.41
	20 s	0.00621	55.6	11.3	0.00000000482	3.78
	40 s	0.00707	58.5	10.1	0.000000000464	4.15
	60 s	0.00727	59.7	9.86	0.00000000483	3.77
	80 s	0.00436	57.6	14.1	0.0000000309	3.41
	100 s	0.00427	58.9	14.0	0.0000000364	3.41
	200 s	0.00453	61.7	12.5	0.0000000231	3.46
	300 s	0.00496	62.4	12.1	0.0000000429	3.32
	400 s	0.00514	63.0	12.0	0.000000217	3.01
	500 s	0.00536	63.6	11.7	0.00000113	2.68
	600 s	0.00534	64.1	11.9	0.00000258	2.59

Table S4.13. Summary of values calculated using SAXS fitting parameters outlined in Table S4.12 for P1₄₀.

Sample		Mean sphere diameter, D_s (nm) $D_s = 2R_s + 4R_g$	Mean aggregation number, N_{agg} $N_{agg} = \frac{\frac{4}{3}\pi R_s^3}{V_s}$
Static		18.6	112
Time-resolved	2 s	Unsatisfactory fit	
	4 s	Unsatisfactory fit	
	6 s	8.05	14.9
	8 s	10.2	32.6
	10 s	11.0	42.2
	20 s	12.1	58.4
	40 s	12.7	67.9
	60 s	12.9	72.2
	80 s	12.5	64.7
	100 s	12.8	69.2
	200 s	13.3	79.6
	300 s	13.5	82.5
	400 s	13.6	84.9
	500 s	13.7	87.2
	600 s	13.8	89.3

Table S4.14. Summary of variable fitting parameters for **P1₆₀** SAXS data.

	Sample	Spherical micelle model			Power law	
		φ_s	R_s (Å)	σ_{R_s} (Å)	B	P
Static		0.0258	87.5	8.64	0	0
Time-resolved	2 s	Unsatisfactory fit				
	4 s	Unsatisfactory fit				
	6 s	Unsatisfactory fit				
	8 s	0.00412	35.5	27.0	0.00571	1.39
	10 s	0.00384	45.6	22.5	0.00489	1.42
	20 s	0.00393	56.7	17.0	0.00394	1.46
	40 s	0.00433	61.3	15.2	0.00428	1.44
	60 s	0.00459	63.5	14.4	0.00273	1.53
	80 s	0.00472	64.8	14.3	0.00353	1.48
	100 s	0.00472	66.0	14.3	0.00216	1.56
	200 s	0.00448	72.1	16.4	0.00268	1.54
	300 s	0.00536	78.9	13.0	0.000917	1.67
	400 s	0.00600	79.5	11.5	0.000134	1.91
	500 s	0.00622	80.4	12.0	0.00876	1.33
	600 s	0.00590	80.1	11.4	0.00000269	2.57

Table S4.15. Summary of values calculated using SAXS fitting parameters outlined in Table S4.14 for **P1₆₀**.

Sample		Mean sphere diameter, D_s (nm) $D_s = 2R_s + 4R_g$	Mean aggregation number, N_{agg} $N_{agg} = \frac{\frac{4}{3}\pi R_s^3}{V_s}$
Static		22.3	152
Time-resolved	2 s	Unsatisfactory fit	
	4 s	Unsatisfactory fit	
	6 s	Unsatisfactory fit	
	8 s	8.09	10.1
	10 s	10.1	21.4
	20 s	12.3	41.2
	40 s	13.3	52.2
	60 s	13.7	57.8
	80 s	14.0	61.6
	100 s	14.2	65.1
	200 s	15.4	84.9
	300 s	16.8	111
	400 s	16.9	113
	500 s	17.1	117
	600 s	17.0	116

Table S4.16. Summary of variable fitting parameters for **P2₂₀** SAXS data.

		Sample	Spherical micelle model			Power law	
			φ_s	R_s (Å)	σ_{R_s} (Å)	B	P
Static			0.0235	44.8	6.60	0	0
Time-resolved	2 s	Unsatisfactory fit					
	4 s	0.00665604	29.6	14.8	0.0000485	2.16	
	6 s	0.00563414	41.8	10.9	0.0000378	2.20	
	8 s	0.00626445	45.7	9.80	0.0000291	2.25	
	10 s	0.00683279	47.7	9.67	0.0000142	2.40	
	20 s	0.010303	50.8	8.55	0.0000126	2.41	
	40 s	0.0130	53.3	7.70	0.0000133	2.37	
	60 s	0.0140	54.6	7.47	0.0000121	2.39	
	80 s	0.0140	55.4	7.50	0.0000101	2.43	
	100 s	0.0144	55.8	7.47	0.00000542	2.54	
	200 s	0.0137	57.3	7.69	0.00000216	2.75	
	300 s	0.0138	58.2	7.76	0.00000741	2.46	
	400 s	0.0139	58.5	7.58	0.00000906	2.50	
	500 s	Unsatisfactory fit					
	600 s	Unsatisfactory fit					

Table S4.17. Summary of values calculated using SAXS fitting parameters outlined in Table S4.16 for P2₂₀.

Sample		Mean sphere diameter, D_s (nm) $D_s = 2R_s + 4R_g$	Mean aggregation number, N_{agg} $N_{agg} = \frac{\frac{4}{3}\pi R_s^3}{V_s}$
Static		11.7	61.0
Time-resolved	2 s	Unsatisfactory fit	
	4 s	6.77	17.7
	6 s	9.20	49.5
	8 s	10.2	64.8
	10 s	10.7	73.7
	20 s	12.2	88.7
	40 s	13.3	103
	60 s	13.7	110
	80 s	13.9	116
	100 s	14.1	118
	200 s	14.3	128
	300 s	14.5	134
	400 s	14.6	136
	500 s	Unsatisfactory fit	
	600 s	Unsatisfactory fit	

Table S4.18. Summary of variable fitting parameters for **P2₄₀** SAXS data.

	Sample	Spherical micelle model			Power law	
		φ_s	R_s (Å)	σ_{R_s} (Å)	B	P
Static		0.0293	80.3	8.15	0	0
Time-resolved	2 s	Unsatisfactory fit				
	4 s	Unsatisfactory fit				
	6 s	0.00660	16.1	29.3	0.320	0.734
	8 s	0.00489	42.8	19.6	0.203	0.827
	10 s	0.00469	49.3	17.0	0.251	0.787
	20 s	0.00511	58.4	13.6	0.0607	1.08
	40 s	0.00578	64.4	64.4	0.0721	1.05
	60 s	0.00614	68.1	11.6	0.0372	1.18
	80 s	0.00621	70.7	11.5	0.0457	1.13
	100 s	0.00640	71.3	10.8	0.106	0.974
	200 s	0.00603	77.2	14.6	0.0168	1.32
	300 s	0.00462	79.0	16.4	0.00209	1.65
	400 s	0.00491	80.7	14.6	0.00224	1.62
	500 s	0.00545	81.1	14.0	0.00217	1.65
	600 s	0.00561	80.7	12.0	0.00307	1.60

Table S4.19. Summary of values calculated using SAXS fitting parameters outlined in Table S4.18 for P2₄₀.

Sample		Mean sphere diameter, D_s (nm) $D_s = 2R_s + 4R_g$	Mean aggregation number, N_{agg} $N_{agg} = \frac{\frac{4}{3}\pi R_s^3}{V_s}$
Static		21.0	176
Time-resolved	2 s	Unsatisfactory fit	
	4 s	Unsatisfactory fit	
	6 s	4.22	1.41
	8 s	9.55	26.5
	10 s	10.9	40.6
	20 s	12.7	67.5
	40 s	13.9	90.4
	60 s	14.6	107
	80 s	15.1	120
	100 s	15.3	123
	200 s	16.4	156
	300 s	16.8	167
	400 s	17.1	178
	500 s	17.2	181
	600 s	17.1	178

Table S4.20. Summary of variable fitting parameters for **P2₆₀** SAXS data.

	Sample	Spherical micelle model			Power law	
		φ_s	R_s (Å)	σ_{R_s} (Å)	B	P
Static		0.0252	108	10.1	0	0
Time-resolved	2 s	Unsatisfactory fit				
	4 s	Unsatisfactory fit				
	6 s	Unsatisfactory fit				
	8 s	0.00438	13.7	37.4	0.0122	1.38
	10 s	0.00410	33.0	32.0	0.00796	1.49
	20 s	0.00349	48.9	23.4	0.0357	1.16
	40 s	0.00338	63.5	17.2	0.0712	1.01
	60 s	0.00380	76.0	17.0	0.0293	1.19
	80 s	0.00397	83.6	16.1	0.108	0.944
	100 s	0.00421	88.9	16.7	0.0478	1.10
	200 s	0.00373	90.3	22.8	0.0110	1.34
	300 s	0.00456	103	22.0	0.0183	1.25
	400 s	0.00478	110	21.4	0.0176	1.28
	500 s	0.00469	115	21.7	0.0270	1.21
	600 s	0.00484	118	21.6	0.0243	1.24

Table S4.21. Summary of values calculated using SAXS fitting parameters outlined in Table S4.20 for P2₆₀.

Sample		Mean sphere diameter, D_s (nm) $D_s = 2R_s + 4R_g$	Mean aggregation number, N_{agg} $N_{agg} = \frac{\frac{4}{3}\pi R_s^3}{V_s}$
Static		27.1	288
Time-resolved	2 s	Unsatisfactory fit	
	4 s	Unsatisfactory fit	
	6 s	Unsatisfactory fit	
	8 s	3.73	0.577
	10 s	7.60	8.12
	20 s	10.8	26.4
	40 s	13.7	58.0
	60 s	16.2	99.1
	80 s	17.7	132
	100 s	18.8	159
	200 s	19.1	166
	300 s	21.7	249
	400 s	23.0	303
	500 s	23.9	341
	600 s	24.6	372

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Chapter 5. Conclusions and Future Work

This thesis has further developed understanding of block copolymer nano-objects formed by ring-opening metathesis polymerisation (ROMPISA), as well as their applicability as biomimetic structures capable of compartmentalisation and material transport.

Initial work aimed to broaden the scope of pH-triggered fusion of ROMPISA polymersomes and demonstrate the generality of the process. The inclusion of a carboxylic acid-functionalised norbornene monomer resulted in the formation of self-assembled structures which displayed a dependence on both solution pH, as well as the core DP. At neutral pH, anionic polymersomes were formed with a spherical morphology. However, at low pH, protonation of the carboxylic acid moiety resulted in the fusion of the nano-objects, forming tubular structures.

Following this, triggered fusion of carboxylic acid-functionalised polymersomes was investigated. A switch to pH 2 following addition of acid resulted in the triggered fusion of spherical nano-objects into fused tubes. Kinetic monitoring provided mechanistic insights into the fusion process, highlighting an inverse rate-dependence for carboxylic acid-containing polymersomes when compared to the amine-functionalised analogues.

In future work, it would be interesting to further explore the mechanism of ROMPISA pH-triggered fusion, with the aim of determining if the differences observed in the mechanisms of positively and negatively charged polymersome fusion are consistent with other pH-sensitive functional groups (e.g. borates). In addition, further analysis by *in situ* SAXS may reveal greater insights into the mechanism of fusion in negatively charged ROMPISA polymersomes.

Previous work by Fielden *et al* demonstrated multistep triggered fusion of ROMPISA polymersomes by using two pH-responsive blocks with different pKa values.¹ As such, this research theme could be further probed using monomers functionalised with a polyacid (e.g. phosphate group).

The synthesis of zwitterionic block copolymers was achieved by incorporation of both carboxylic acid- and amine-functionalised blocks within a single chain. The dual, pH-responsive properties of the resulting polymersomes was demonstrated by sequential pH switching, with block order and microstructure shown as key determinants of final particle morphology.

Possible future experiments into zwitterionic polymersomes could involve a greater number of pH switching cycles and observing any further changes to particle morphology or size. In addition, the inclusion of zwitterionic monomers (e.g. amino acids), such as those reported by Tew *et al*, would be interesting areas to explore.²

The second focus of this thesis was to explore the charge-mediated fusion of polymersomes formed by ROMPISA. Cationic and anionic polymersomes of varying core DP were mixed, resulting in different morphologies formed, including linear tubes, branched structures, and irregular clusters. Final nano-object morphology was shown to be affected by disparity in original polymersome size as well as core DP equivalence. In addition, fusion was observed when polymersomes below the critical core DP to fuse were mixed, indicating electrostatic charge attraction can be utilised as an energetic driving force for polymersome fusion.

To further investigate charge mediated ROMPISA polymersome fusion, functional groups that are charged but not pH-sensitive (e.g. quaternary amine, sulphonate) should be explored. This

would eliminate any impact from a change in pH upon particle mixing, and ensure fusion is caused solely by the electrostatic attraction between oppositely charged polymersomes.

The mode of fusion was investigated using a model system of ROMPISA polymersomes tagged with fluorescent dyes that formed a FRET pair when brought into close proximity. Fluorescence analysis displayed a greater FRET response when the particles were seen to fuse compared to an unfused system suggesting fusion is occurring heterogeneously, where fusion takes place between the two different particles.

Next steps in exploring the mode fusion would focus on improving the model system, particularly the choice of fluorescent dyes. Although there is spectral overlap between pyrene emission and naphthalimide excitation, resulting in a FRET when in close proximity, the presence of pyrene excimers greatly influences the resulting emission intensity. As such, alternative FRET pairs (e.g. fluorescein and rhodamine, Cyanine3 and Cyanine5) should be explored. Following this, the inclusion of FRET dyes within more complex systems described in this thesis (e.g. pH-triggered or charge-mediated fusion) would allow for a greater understanding of their mechanisms.

In addition, direct visualisation of the resulting fused structures would be of huge benefit in confirming the mode of fusion taking place. Recently developed super-resolution microscopy techniques, such as structured illumination microscopy (SIM) and stochastic optical reconstruction microscopy (STORM), can achieve resolutions of as little as 5 nm.³⁻⁵ As such, these techniques would reveal further information about the mode of fusion, as well as the final morphology of the fused structures.

Finally, the encapsulation efficiency of ROMPISA nano-objects, and the impact of the mechanism of formation on this property, was probed. A range of water-soluble cargos were shown to not be successfully encapsulated within ROMPISA nano-objects during self-assembly, irrespective of size or concentration. As such, this suggests the nano-objects form *via* a mechanism that precludes encapsulation. *In situ* SAXS analysis indicated a spherical morphology was present throughout the ROMPISA process, with no formation of anisotropic particles. Mechanism I starts with the formation of micelles which grow into anisotropic bilayers before enclosure to form a vesicle, permitting encapsulation within the lumen. In contrast, Mechanism II proceeds *via* the inversion of unimers within a micelle to form a semi-vesicle (a vesicle with no lumen) before solvent molecules diffuse through the bilayer to form a vesicle. As such, the inability to encapsulate water-soluble cargo and the presence of a spherical morphology during self-assembly tentatively suggest ROMPISA nano-objects form by Mechanism II.

This theory could be further investigated by using a fluorescent dye to label the hydrophobic end of the polymer chain. The fluorescent emission of the dye is impacted by its environment (e.g. hydrophobicity or solvation) and so can report on its environment during the self-assembly process, as utilised by Rho *et al* in a recent report.⁶ Due to the rapid formation of nanostructures, conventional techniques (e.g. TEM or DLS) are unable to determine morphology in the early stages of ROMPISA. However, *in situ* fluorescent monitoring would allow for greater elucidation of the morphological transitions that are taking place and thus the overall mechanism of formation.

Due to the limitations of TEM and DLS (as discussed in the introduction), supplementary characterisation of the nanostructures formed *via* ROMPISA should be performed to gain further evidence of the polymersome structure. For example, cryogenic TEM (cryo-TEM) allows for nanostructures to be frozen in their native solution state, reducing the impact of dehydration effects. Additionally, electron energy loss spectroscopy (EELS) can be used in combination with TEM to provide atomic-level chemical and structural characterisation. This combination of techniques has a spatial resolution of nanometres and so can differentiate between the corona and core blocks of the polymer and determine the presence of a polymersome bilayer in the ROMPISA nanostructure.

In summary, research focussed on increasing the breadth of selectivity in triggered fusion (e.g. light-responsive, DNA-mediated), as well as generating a greater understanding of the mechanisms taking place, would allow for highly complex, autonomous systems to be realised. This would lead to the creation of a toolbox for directing various polymersome fusion pathways and have impact in “real-world” applications, such as reagent trafficking, switchable catalysis cascades and biomedical nanotechnology.

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