Electronic Supplementary Information

Fluorescent Styrene Maleic Acid Copolymers to Facilitate Membrane Protein Studies in Lipid Nanodiscs

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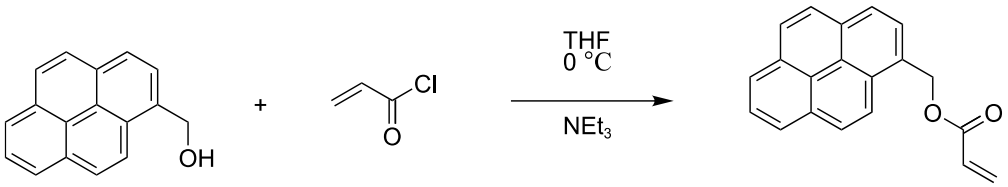
1.0 Copolymer Synthesis

1.1 Materials

Prior to polymerisation, styrene (Merck, purity ≥ 99%) was passed through a disposable column (Merck) to remove the inhibitor, 4-tert-butylcatechol. The comonomer, maleic anhydride (MAnh) (puriss, purity ≥ 99%), the initiator, 2,2'-azobis(2-methylpropionitrile (AIBN), the RAFT agent, 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DDMAT) (purity 98%, HPLC grade), and the solvent, 1,4-dioxane were purchased from Merck and used as received. The commercial SMA variant, *SMA 2000*, was provided by Cray Valley. All other solvents used were purchased from Merck and used as received.

1.2 Synthesis of 1-pyrenemethyl acrylate (PmAc)

Insertion of an active C=C bond onto pyrene (*Scheme S1*) was adapted from the work of Lou *et al*. (2004)[1]. Briefly, 1-pyrenemethanol (0.5 g, 0.0022 mol) and triethylamine (purity ≥ 99%, 0.1 ml, 0.0065 mol) were dissolved in 25 ml THF (GPC grade) before dropwise addition of acryloyl chloride (purity ≥ 97%, 400 ppm phenothiazine stabiliser, 0.6 ml, 0.0065 mol) at 0 °C. The solution was stirred for 12 hours and allowed to reach room temperature. THF was evaporated under vacuum and the solid residue dissolved in 30 ml dichloromethane. The solution was washed with 10 ml of 1.0 M HCl in a separating funnel, followed by aqueous sodium hydrogen carbonate (10 ml, 1.0 M) and finally water. After drying with magnesium sulphate, the resulting solution was filtered and residual solvent removed by rotary evaporation. The resulting yellow solid was dissolved in 0.5 ml THF and precipitated from 2 ml methanol. This was left for 20 hours at -20 °C, before being filtered. Reprecipitation was then repeated before the final solid product was collected. The NMR spectrum was satisfactory, albeit showing some levels of THF and methanol. These would not interfere with, and would be removed, during work up of the succeeding polymerization reactions.



Scheme S1

1.3 RAFT Polymerisation of SMAnh Copolymers

RAFT polymerisation protocols were adapted from those described by Harrison and Wooley.[2] Reagents were sealed in a round bottomed flask in the quantities outlined in *Table S1* and were deoxygenated with nitrogen before three freeze-pump-thaw cycles. Containers were covered with aluminium foil to exclude light before heating to 60 °C for 24 hours. Copolymers were precipitated in 500 ml ice-cold diethyl ether before drying in an oven (~ 40 °C) overnight.

In the case of the fluorescent SMA variants, *SMA-VA* and *SMA-Py*, the procedure was the same except for the inclusion of 9-vinylanthracene (purity ≥ 97%, Merck) or 1-pyrenemethyl acrylate (synthesised as in 1.2) at the indicated mass percentage of the feed (*Table S1*).

Table S1: Reagent quantities for RAFT copolymerisation of SMAnh.

Sample / Abbreviation	Reagent	Mass / g	Mol Reagent	Predicted Molecular Weight $M_{n(pre)}$ / kDa*
SMAnh R	Styrene	1.0003	9.60×10^{-3}	6
	Maleic anhydride	0.4039	4.12×10^{-3}	
	AIBN	0.0433	2.64×10^{-4}	
	DDMAT	0.0905	2.48×10^{-4}	
	1,4-dioxane	3.0000	3.40×10^{-2}	
SMAnh-VA0.1	Styrene	1.0003	9.60×10^{-3}	6
	Maleic anhydride	0.4039	4.12×10^{-3}	
	AIBN	0.0433	2.64×10^{-4}	
	DDMAT	0.0905	2.48×10^{-4}	
	1,4-dioxane	3.0000	3.40×10^{-2}	
	9-vinylanthracene	0.0020	9.79×10^{-6}	
SMAnh-VA0.01	Styrene	1.0003	9.60×10^{-3}	6
	Maleic anhydride	0.4039	4.12×10^{-3}	
	AIBN	0.0433	2.64×10^{-4}	
	DDMAT	0.0905	2.48×10^{-4}	
	1,4-dioxane	3.0000	3.40×10^{-2}	
	9-vinylanthracene	0.0002	9.79×10^{-7}	
SMAnh-Py0.01	Styrene	1.0003	9.60×10^{-3}	6
	Maleic anhydride	0.4039	4.12×10^{-3}	
	AIBN	0.0433	2.64×10^{-4}	
	DDMAT	0.0905	2.48×10^{-4}	
	1,4-dioxane	3.0000	3.40×10^{-2}	
	PmAc	0.0001	3.50×10^{-7}	

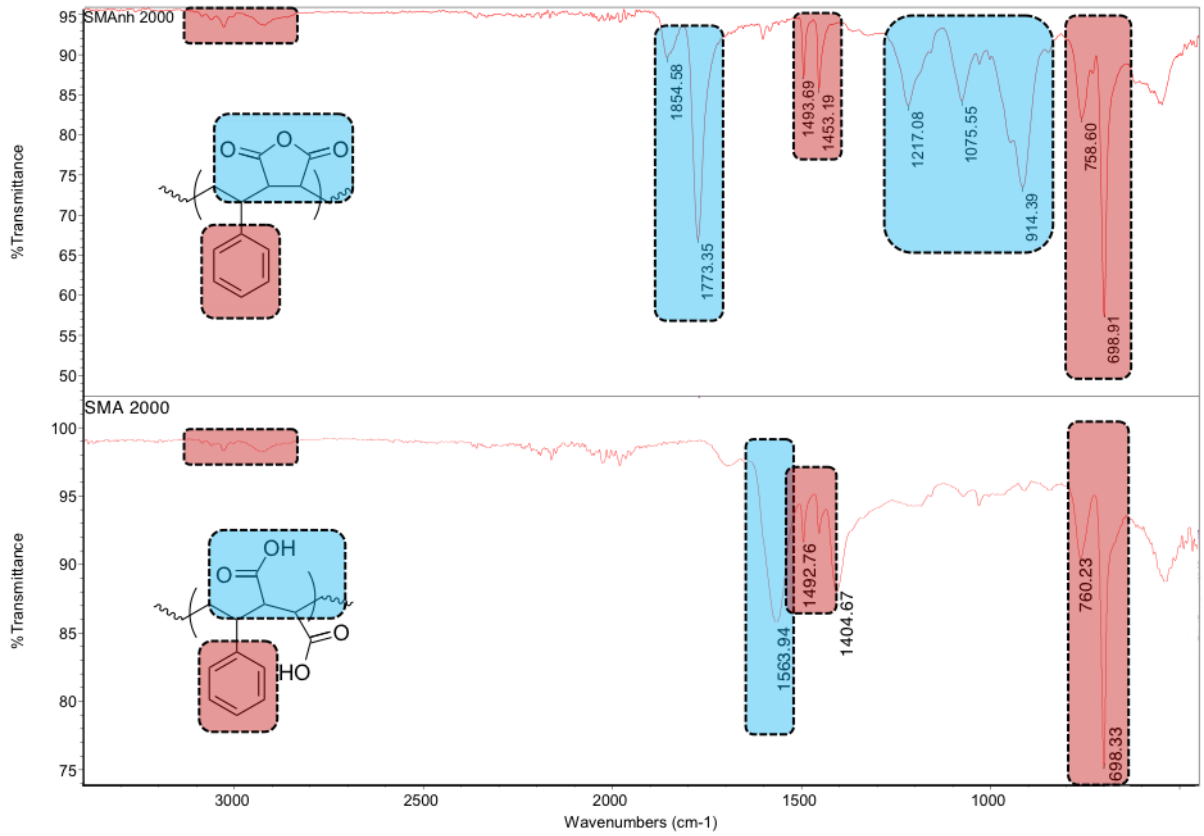
**Estimated by:* $M_{n(pre)} = ((mol_{(Sty)} \times M_{r(Sty)} \times conversion) / mol_{(DDMAT))} + ((mol_{(MAnh)} \times M_{r(MAnh)} \times conversion) / mol_{(DDMAT))} + M_{r(DDMAT)}$.

1.4 Hydrolysis of *SMAnh* to *SMA*

Following the procedure outlined by Hall *et al.*,[3] *SMAnh* was hydrolysed to *SMA* using reflux under basic conditions. Typically, a 10% (wt./ vol.) polymer solution was prepared in 1.0 M NaOH (aq) and heated under reflux for 2 hours. The solution was then cooled to room temperature before precipitation of *SMA* by acidification to pH 3.0 with 4.0 M aqueous HCl. The resulting mixture was centrifuged at 8000 rpm using an *Eppendorf 5804R* centrifuge for 15 minutes at 21 °C. The supernatant was removed and the polymer pellet washed with ultrapure water and again recovered by centrifugation. The procedure was repeated a further three times. The pellet was then dissolved in 0.6 M NaOH before repeating the precipitation and washing procedure. The final precipitate was then dissolved in a minimal amount of 0.6 M NaOH and adjusted to pH 8.0 before freeze drying (*Virtis SP Scientific*) for a minimum of 24 hours.

Conversion of *MAnh* to *MA* was monitored using FTIR measurements which were conducted on a *Perkin Elmer* ATR desktop spectrometer with solid-state polymer samples at room temperature between 500 – 4000 cm^{-1} recording 16 scans with a resolution of 1 cm^{-1}. Example spectra indicating the successful conversion of *SMAnh 2000* to *SMA 2000* are shown in *Figure S1*. Spectra for other polymers showed similar reaction.

(a)



(b)

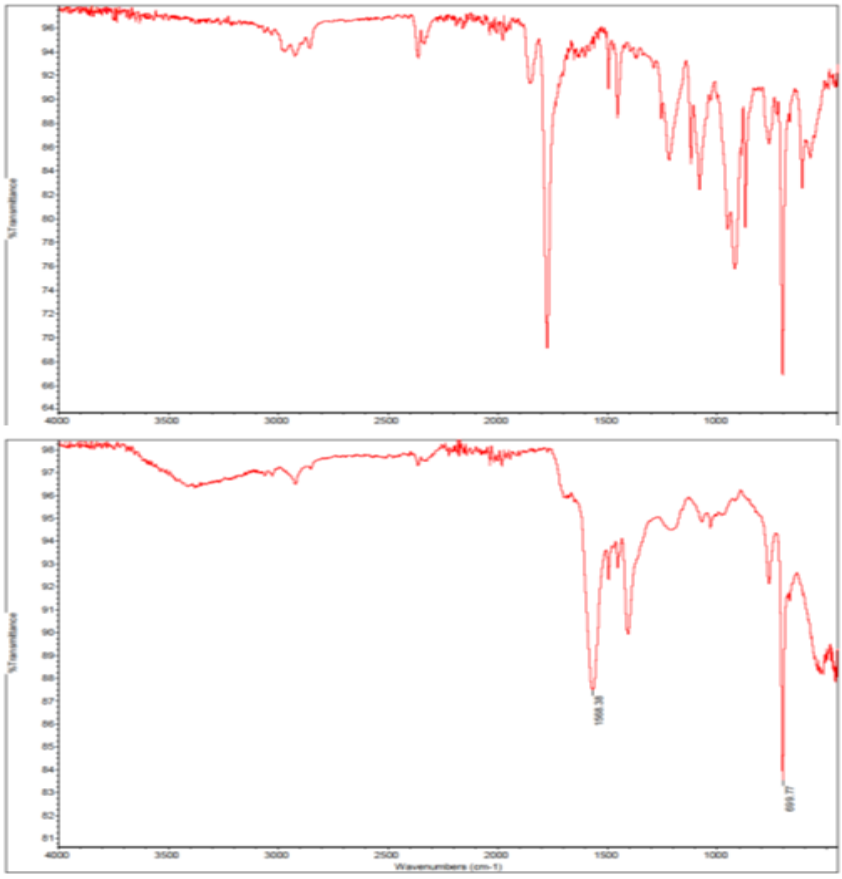


Figure S1: (a) FTIR spectra of (Top) SMAnh 2000 and (Below) SMA 2000, highlighting changes to those peaks pertaining to (blue) maleic anhydride/acid during hydrolysis and how those relating to (red) styrene remain unchanged. The indicated wavenumbers are assigned by the spectrometer data system and are not meant to indicate the resolution of the peaks. (b) Spectra of (Top) anhydride variant SMAnh-VA0.1 and (Below) acid variant SMA-VA0.1.

2.0 Copolymer Characterisation

2.1 ^{1}H & ^{13}C NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on an *Agilent* 500 MHz spectrometer at room temperature and were subsequently analysed using *Mestrelab MNova 11.0* software. Polymer samples were prepared by dissolving in d_6-acetone (*SMAnh*) or D2O (*SMA*) at concentrations of ~40 mg ml^{-1}.

An example ^{1}H spectrum for *SMA-VA0.01* is show in in *Figure S2*. After baseline correction, peak integration of those associated with *Sty* (δ = 7.60-6.05) versus *MAnh* (δ = 3.05-3.50) gives a monomer ratio of 2:1. Similar spectra were obtained for all polymers presented. Anthracene and pyrene units could not be identified by NMR (nor FTIR) due to the low levels of fluorophore incorporation and also their signals being masked by styrenic peaks which dominate the aromatic ranges of NMR chemical

shift. This meant quantitative determination of fluorophore incorporation was not possible, and instead fluorophore content was estimated from conversion (See *Table S2*).

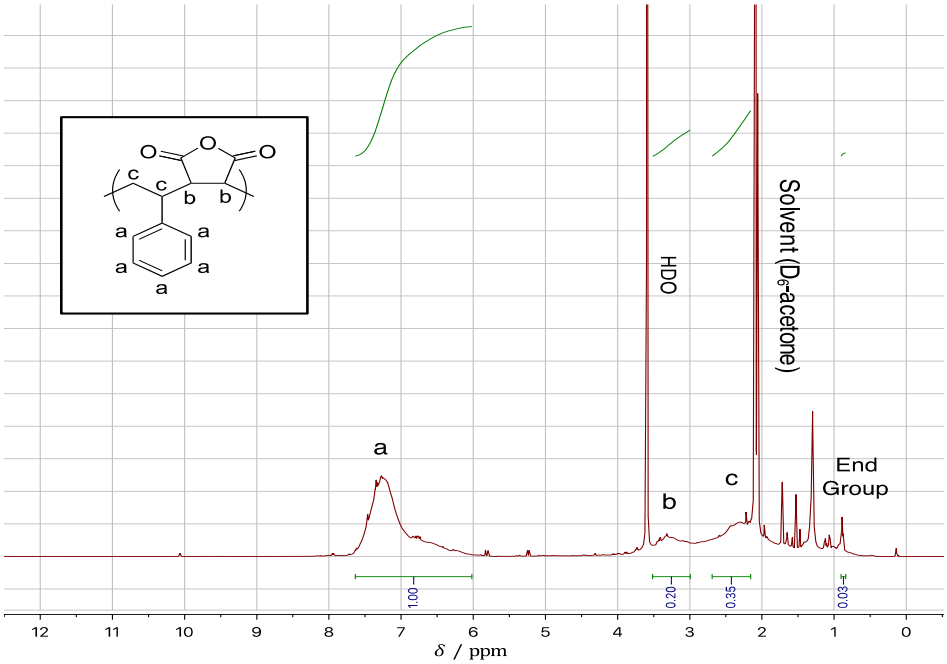


Figure S2: ^{1}H NMR spectrum of *SMAnh-VA0.01* [(D_6-acetone): δ 7.60-6.05 (5H, broad, H_a), 3.05-3.50 (2H, broad, H_b), 2.15-2.70 (3H, broad, H_c), 0.87-0.90 (m, DDMAT end group)].

SMAnh 2000 is synthesised by 'starved-feed' radical polymerization and so has a random arrangement of *Sty* and *MAnh* units. By contrast, the use of RAFT to produce *SMAnh R* results in a diblock structure. Initially, chains will consist of alternating *Sty* and *MAnh* groups. As polymerization proceeds and the *MAnh* is consumed, a block of homo-polystyrene forms. There will be a small 'transition' or 'gradient' region between these blocks.

^{13}C NMR spectra were used to confirm the presence of block architectures[8,9]. Fig. S3 compares spectra from the commercial *SMAnh 2000* and *SMAnh R*. It was previously shown[8] that peaks at 36.3 (*f*) and 40.5 ppm (*h*) refer to the alternating block, and the peaks at 42.0 (*e*) and 51.8 ppm (*g*), the styrene homoblock.[4] For *SMAnh 2000* all peaks are largely broadened, with the 36.3 ppm (*alt*) peak missing as expected for a random structure.

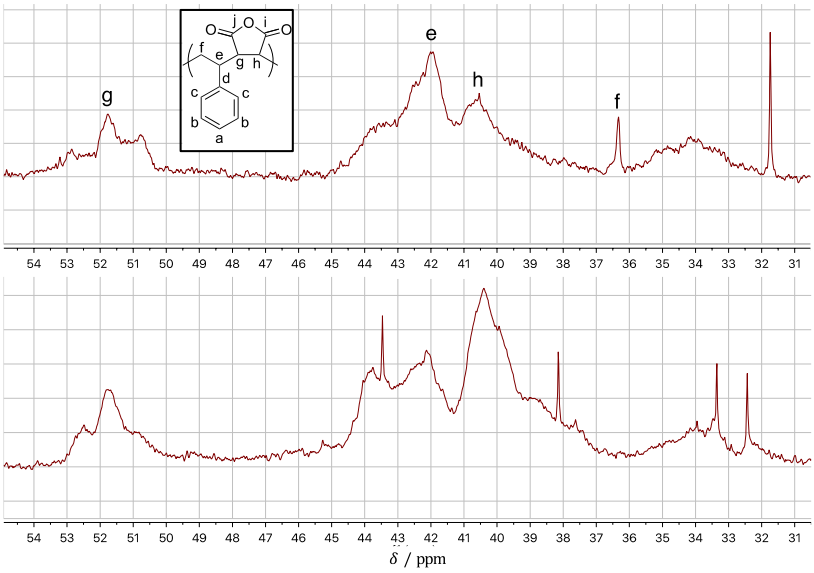


Figure S3: ^{13}C NMR spectra for (Top) *SMAnh R* and (Bottom) *SMAnh 2000.*

2.2 Gel Permeation Chromatography

SMAnh polymer molecular weights were estimated by GPC using an *Agilent GPC 1260 Infinity* chromatograph using two PLgel 5µM MIXED-D 30 cm x 7.5 mm columns with a guard column PLgel 5 µm MIXED Guard 50 x 7.5 mm. The column oven was maintained at 35 °C, with GPC-grade THF as the eluent at a flow rate of 1.00 mL/min and refractive index detection and polymer concentrations between 1.0 – 2.0 mg/mL. The system was calibrated against 12 narrow molecular weight polystyrene standards with a range of Mw from 1050 Da to 2650 kDa. Chromatograms (Figure S4) were subsequently analysed in *Agilent GPC/SEC* software to extract M_n and PDI values (Table S2).

As expected, the RAFT copolymers synthesised had a low PDI (1.2-1.3) in comparison to the commercial variant (1.8). All copolymers had similar M_n values and hence are assumed to present similar block lengths. This allows the effect of fluorophore inclusion to be assessed, free from convolution with effects arising from polymer chain length.

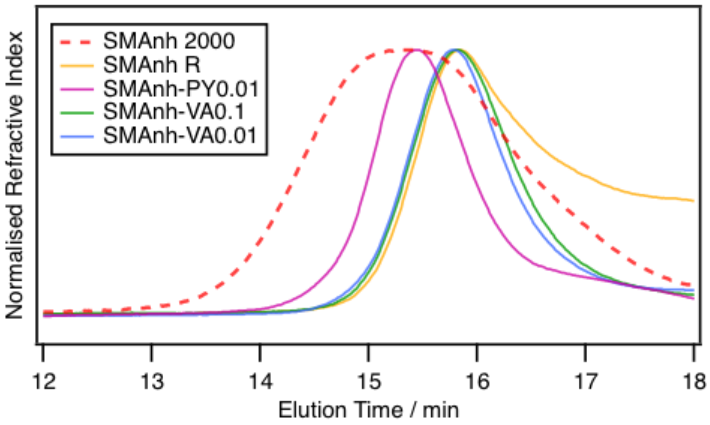


Figure S4: Normalised GPC chromatograms of *SMAnh* polymers.

Table S2: Properties of *SMAnh* polymers

Sample / Abbreviation	Conversion / %	M_n / kDa	PDI	DP_n Sty*	DP_n MAnh*	Length Sty Homoblock**
SMAnh (R)	64	3.220	1.19	21	11	10
SMAnh-VA0.1	62.9	3.199	1.27	20	11	9
SMAnh-VA0.01	56.0	3.426	1.24	22	12	10
SMAnh-PY0.01	87.0	5.141	1.16	33	17	16
SMAnh 2000	-	4.000	1.80	-	-	-

$^{*}\ DP = \frac{M_n \times Monomer\ Ratio\ (NMR)}{M_{monomer}}$ $^{**}Length\ Sty\ Homoblock = DP_nSty - DP_nMAnh$

3.0 Nanodisc Formation and Scattering

3.1 Materials and Nanodisc Preparation

The lipid, 1,2-dimystoyl-sn-glycero-3-phosphocholine (DMPC) (purity ≥ 99%), was purchased from Merck and mono and dibasic sodium phosphate (purity ≥ 99%) from Acros Organics. A 50 mM phosphate buffer solution (PBS) was prepared by mixing 0.1 M aqueous monobasic sodium phosphate (2.65 ml, 2.65×10^{-4} mol) and dibasic sodium phosphate (47.35 ml, 4.735×10^{-3} mol) and making up to 100 ml with pure water (18.2 ΩM). NaCl (1.1688 g, 0.02 mol) was added to give a 0.2 M salt concentration. This produced a PBS stabilised at pH = 8.0, representing physiological conditions and within the pH range SMALPs nanodiscs are stable.

DMPC (0.005 g, 7.38×10^{-6} mol), was added to 0.679 ml PBS and dispersed by sonication in two 10-second bursts, separated by a 15-second rest period to prevent overheating. 0.015 g of SMA in 0.231 ml PBS was then added, producing a nanodisc solution containing 1.65% (wt.) polymer and 0.55% (wt.) lipid. An immediate gauge of successful nanodisc formation can be observed from the loss of turbidity of the lipid suspension upon the introduction of polymer.

The model membrane protein, gramicidin, was incorporated into nanodisc from vesicles prepared by the well-established thin film method, described by Rawat *et al.* (2004).[5] This was achieved by first dissolving DMPC (0.005 g, 7.38×10^{-6} mol) in a minimum volume of 1:1 chloroform:methanol and gramicidin (0.0004 g, 2.13×10^{-7} mol) in a minimal volume of methanol, before mixing. A few drops of chloroform were added before the solution was dried by rotary evaporation at 40 °C until only a residual film remained. This film was then swelled with 1 ml warm (30 °C) PBS and briefly vortexed to create a homogenous suspension. This was sonicated and used with polymer solutions as described above.

3.2 Dynamic Light Scattering (DLS) and Aggregation Behaviour

DLS was conducted using a *Malvern Zetasizer Nanoseries* at $\theta = 173$ ° (backscattering) and $\lambda = 633$ nm using disposable cuvettes. Refractive index and viscosity values were set from poly(styrene-*alt*-maleic acid) in phosphate buffer solution (50 mM, 0.2 M NaCl). Suspensions were diluted to a concentration

of 0.1% wt. polymer to ensure an infinite dilution regime. Prior to measurement, suspensions were passed through a 0.45 μm *Millex Millipore* membrane syringe-driven filter to remove any potential contaminant scatterers.

During investigation it became apparent that RAFT-made SMA copolymers were able to form higher-order, polymer-only aggregates in solution whereas the commercial variant, SMA 2000, did not. This may be reconciled through the estimation of the diameter of a Gaussian coil for SMA 2000 using Equations 1-2:

$$\text{Diameter} = 2R_g = \frac{R^{0.5}}{6} \quad (\mathbf{1})$$

$$R = 1.54 \times N^{0.6} \quad (\mathbf{2})$$

where R_g is the radius of gyration and N is the number of monomer units in the polymer chain, estimated from *Mn*. This provides an estimated diameter of 0.62 nm and, given that DLS overestimates size, the 1-2 nm diameters found from DLS (see main text) are in good agreement that SMA 2000 indeed exists as single chains. For RAFT-made SMA, a threshold concentration, approximately 0.05% wt., was found for aggregate formation. Further investigation into this behaviour is ongoing to investigate the implications for wider nanodisc research.

3.3 Small Angle X-ray Scattering (SAXS) of Nanodiscs

SAXS data were collected *in vacuo* on a *Xenocs NanoInXider* instrument using a CuK X-ray tube source. Nanodisc samples were sealed in 1 mm capillary tubes and experiments conducted at the *Materials Characterisation Laborato*ry at the *ISIS Neutron and Muon Source* (Rutherford Appleton Laboratory). Data were reduced using *Foxtrot*, and fit to parameters using a core shell bicelle model (Fig. S5) within *SASview* software. Parameters held constant during fitting are given in Table S3.

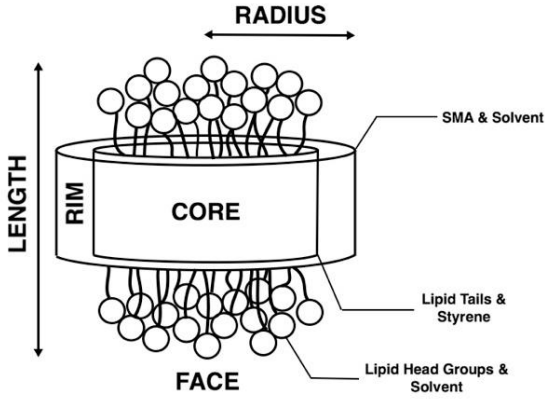


Figure S5: Schematic of core shell bicelle model in the context of fitting SAXS data from nanodisc suspensions.

Parameters for the models used to fit data are presented in Table S4. The data from SMA 2000 illustrates the characteristic double hump pattern expected from nanodisc samples (Fig. S6). Here, SAXS confirms nanodisc morphology with a total disc diameter of 6.4 nm, in good agreement with DLS results (see main text). No inclusion of scattering from e.g. polymer aggregates was needed to satisfactorily fit the data for SMA2000. By contrast, data from SMA-VA0.1 required that this model be combined with a secondary cylindrical aggregate model to account for signs of polymer aggregation; the steeper gradient seen at low q is indicative of this. Whilst the double hump pattern was less

apparent in this data, the satisfactory fit suggests that SMALP nanodiscs were present in addition to this aggregation. Here, the nanodiscs were found to be 17.8 nm in diameter. This was lower than that found by DLS (24 nm), although DLS is expected to overestimate absolute particle size due to interrogation of the hydrodynamic radius as well as interference arising from the fluorescent sample. It is still unclear what the aggregate model represents in the context of this nanodisc suspension, *i.e.* whether the aggregates are between individual nanodiscs or are solely composed of polymer. Ongoing neutron scattering work aims to exploit contrast to elucidate this behaviour further.

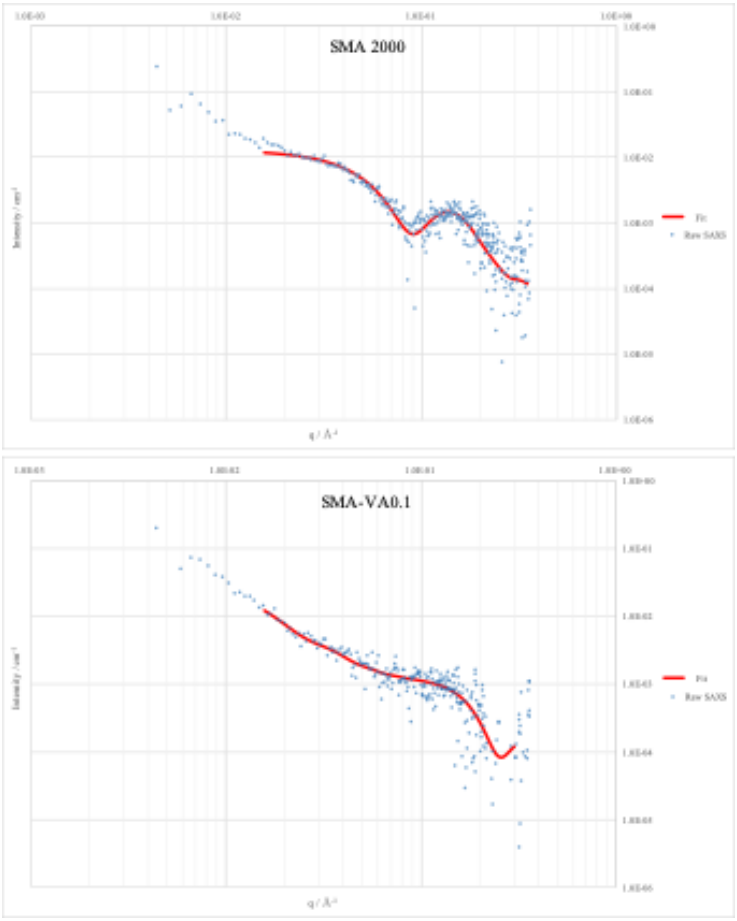


Figure S6: SAXS data and fit for nanodiscs from (Top) *SMA 2000* and (Bottom) *SMA-VA0.1*

Table S3: Model parameters held constant during fitting for data from nanodisc suspensions.

Fixed Property	Value
SLD solvent	9.46×10^{-6} Å^{-2}
SLD face*	10.30×10^{-6} Å^{-2}
Face thickness**	8.00 Å
Length**	28.00 Å

* Based on 57% hydration of lipid head groups[6]
** Set values from literature[6, 7]

Table S4: Properties for nanodiscs as obtained from SAXS measurements. *

Polymer in Nanodisc	Property	Value	χ^{2**}
SMA 2000	Radius◊ Rim Thickness◊ SLD Core SLD Rim PDI*** Total Disc Diameter****	18.8 ± 0.8 Å 13 ± 1 Å $7.8 \pm 0.1 \times 10^{-6}$ Å^{-2} $11.1 \pm 0.1 \times 10^{-6}$ Å^{-2} 0.245 63.6 ± 3.6 Å	2.12
SMA-VA0.1	**Nanodisc** Radius◊ Rim Thickness◊ SLD Core SLD Rim PDI*** Total Disc Diameter**** **Cylindrical Aggregate** Radius Length SLD	 79 ± 1 Å 10 ± 1 Å $8.20 \pm 0.01 \times 10^{-6}$ Å^{-2} $10.0 \pm 0.6 \times 10^{-6}$ Å^{-2} 0.240 178 ± 4 Å 160 ± 10 Å 30 ± 4 Å $11.5 \pm 0.1 \times 10^{-6}$ Å^{-2}	2.97

** ◊ indicates that values and error were fitted by SASview software.*

All other values were fit by trial and error; error taken as change in value to increase χ^2 by 0.1.

***Lower value denotes higher fit accuracy*

****Obtained from DLS data and fixed.*

*****Total Diameter = 2 × (Radius + Rim Thickness)*

4.0 Fluorescence Spectra

All measurements were taken using an *Agilent Cary Eclipse Fluorescence Spectrometer* (slit width = 5 nm) with quartz cuvettes. Fig. S7a-b demonstrates the ability to excite the VA fluorophores within *SMA-VA0.1* at either 370 nm (VA excitation) or 260 nm (styrene excitation), indicating that both units coexist on the same chains. Fig. S7c compares the emission (excitation 370 nm) between *SMA-VA0.01* with *SMA R*, a variant synthesised in the absence of fluorophores, highlighting the lack of emission when excited at this wavelength. Fig. S7d shows how at equivalent concentrations (10^{-7} M), *SMA-VA0.1* has a higher intensity of emission compared to *SMA-VA0.01*, due to its higher fluorophore content.

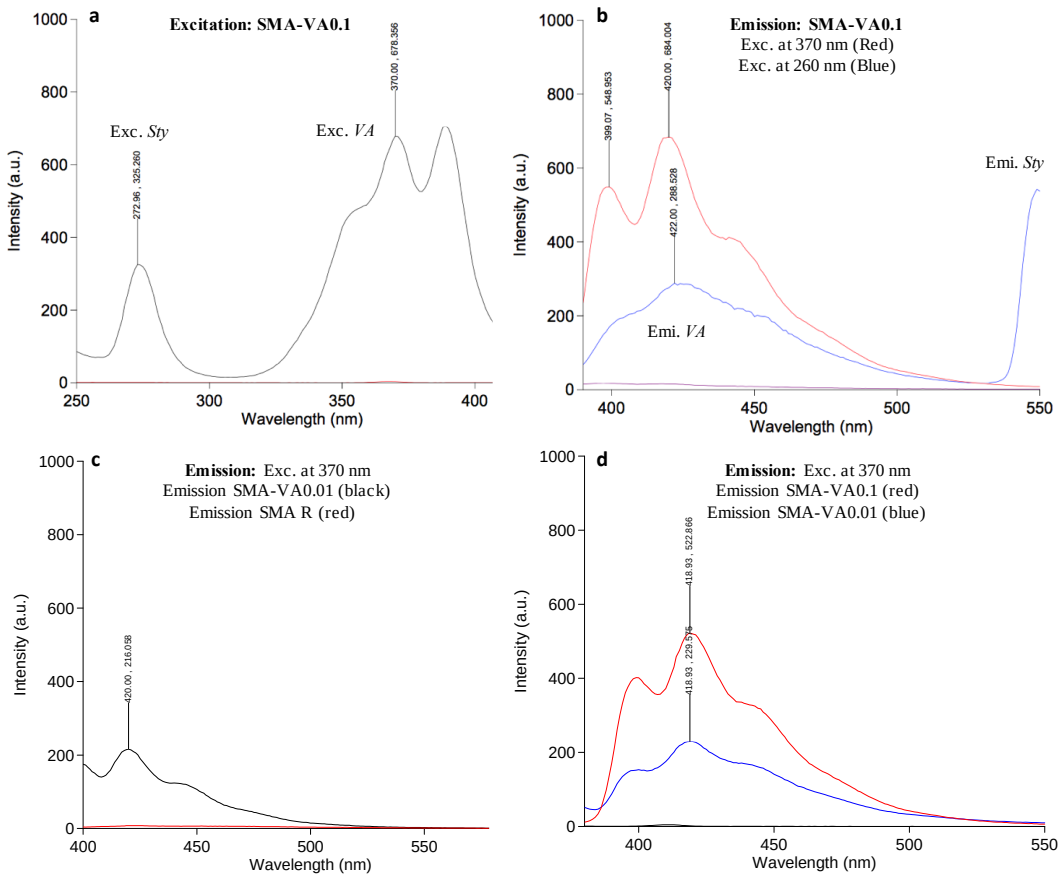


Figure S7: (a) Excitation spectra for *SMA-VA0.1* and (b) emission spectra upon excitation at 370 nm (VA, red) and 260 nm (styrene, blue). (c) Emission spectra of *SMA-VA0.01*(black) vs. SMA R (red) when excited at 370 nm, highlighting the lack of emission for *SMA R*. (d) Emission spectra for *SMA-VA0.1* (red) vs. *SMA-VA0.01* (blue) when excited at 370 nm at equivalent concentrations, highlighting the greater emission intensity of *SMA-VA0.1* due to its higher fluorophore content.

Fig. S8 compares the emission of *SMA-VA0.01* and *SMA-PY0.01* copolymers in various phases. Due to the aggregation caused quenching (ACQ) behaviour of the fluorophores used, when the copolymer aggregates in aqueous solutions, this causes a large decrease in emission intensity which returns when aggregates dissociate upon incubation with lipids during nanodisc formation.

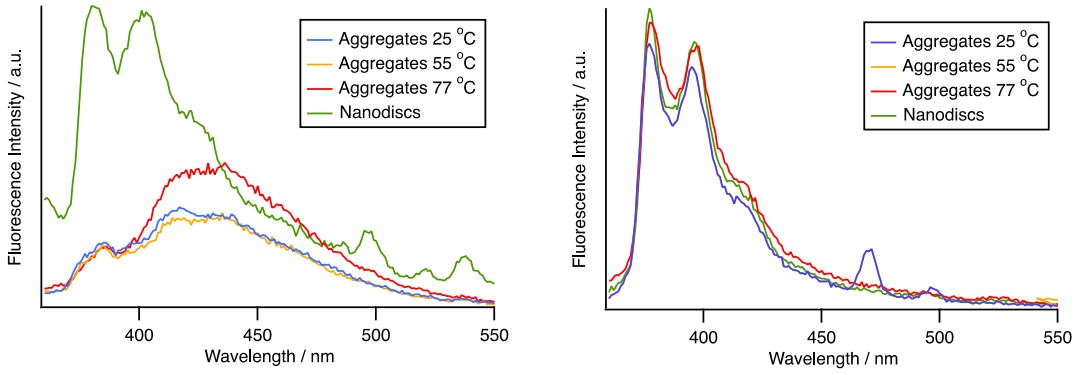


Figure S8: Emission spectra for (Left) *SMA-VA0.01* (excited at 370 nm) and (Right) *SMA-PY0.01* (excited at 340 nm) copolymers in various structural forms, across a range of temperatures.

4.0 References

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