Electronic Supplementary Material (ESI) for Chemical Communications.

Supplemental Information for:

Mapping the supramolecular assembly space of poly(sarcosine)-b-poly(propylene sulfide) using a combinatorial copolymer library

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1. General considerations

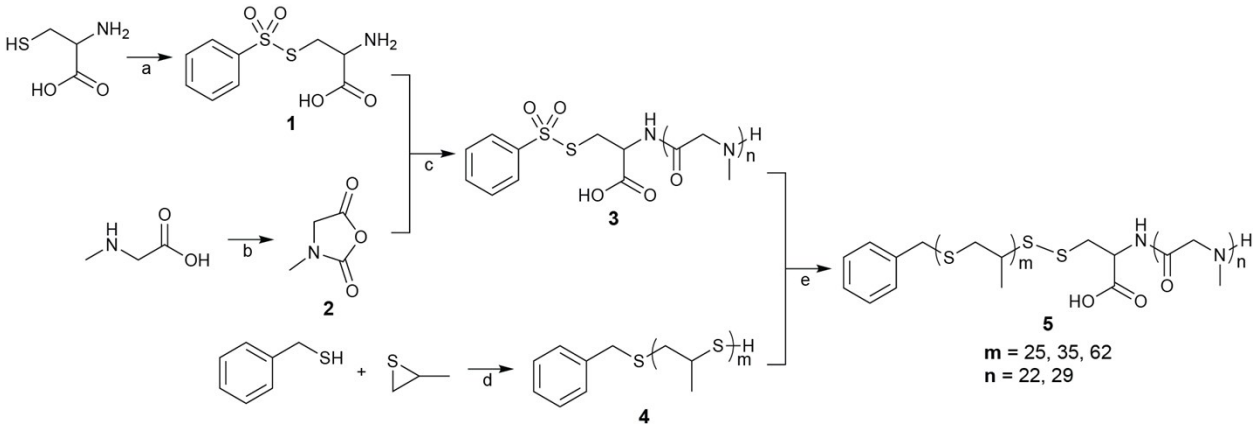
Materials. Unless indicated below, chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and used as received. Hexanes, methanol, ethyl eosin, and DiI were purchased from Fisher Scientific (Pittsburgh, PA, USA). Propylene sulfide was purchased from TCI Chemicals (Boston, MA, USA).

General conditions. Unless otherwise indicated, synthetic procedures were performed under inert conditions, with freshly opened anhydrous solvents and a nitrogen atmosphere. After workup, products were used immediately or quickly transferred to vials kept under nitrogen and stored in the -20 C freezer until use. Special care should be taken when preparing NCA derivatives given its extreme sensitivity to decomposition or ring-opening.

Safety. Phosgene and its derivatives are EXTREMELY HAZARDOUS to human health at any scale. Please take all appropriate safety measures into consideration when planning, handling, and disposing of these chemicals and any materials that have come into contact with them.

2. Experimental procedures

a. Synthesis



Scheme S1 Synthesis of PSarc-PPS diblock copolymer (5). *Conditions*: (a) sodium nitrite, water, 0ºC, 1.5 h; benzenesulfonic acid sodium salt, 0ºC to r.t., 4 h. (b) diphosgene, THF, 55-60ºC, 4 h. (c) DMF, r.t., 96 h. (d) NaOMe, DMF, 20ºC, 15m; AcOH, r.t., 2 min. (e) base, MeOH, r.t., o/n.

Preparation of S-phenylthiosulfonyl-cysteine (FTS-Cys, **1**)

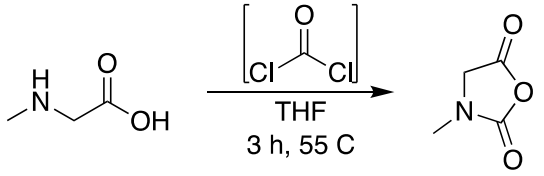
The FTS-Cys initiator was prepared as previously described.[1] Briefly, cystamine hydrocholoride monohydrate (1.5 g, 8.5 mmol) was dissolved in ice-cold 2 M hydrochloric acid to a concentration of 1 M (8.5 mL). In a separate container, sodium nitrite (600 mg, 8.5 mmol) was dissolved in 5 mL ice-cold milli-Q water and added dropwise to the stirring cysteine solution. Benzenesulfinic acid sodium salt (3.5 g, 21.3 mmol) was added to the red solution in two parts to form a solid precipitate, first after 1.5 h (2.8 g, 17.1 mmol) and again after 2 h (700 mg, 4.25 mmol). The

reaction was stirred for an additional 2 hours and stored at 4 C overnight. The solid precipitate was collected via filtration, washed with ice-cold milli-Q water, and recrystallized from water yielding off-white fluffy needle-like crystals (1.07 g, 47.8% yield).

^{1}H NMR (500 MHz, DMSO-d_6 + TFA): δ (ppm) = 7.96 (d, 2H), 7.71 (t, 1H), 7.80 (t, 2H), 4.22 (dd, 1H), 3.46 (dd, 2H).

^{13}C NMR (126 MHz, DMSO-d_6 + TFA): δ (ppm) = 169.01, 143.80, 135.09, 130.37, 127.22, 51.82, 35.04.

Preparation of sarcosine N-carboxyanhydride (SarcNCA, **2**)

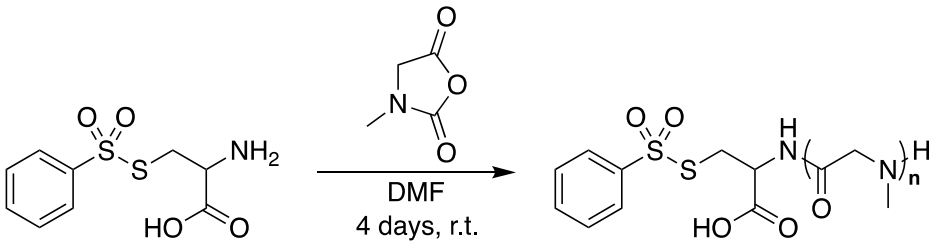


The SarcNCA monomer was prepared as previously described[2] with the following modifications. Briefly, sarcosine (2 g, 22.5 mmol) previously dried under high vacuum at 150 C for 3 days was added to 20 mL anhydrous THF. The suspension was set in an inert atmosphere while slowly heated to reflux before carefully adding triphosgene (2.7 g, 8.9 mmol) or diphosgene (1.6 mL, 13.5 mmol). After 3 hours, the mixture became clear and the reaction was cooled to room temperature. Nitrogen was bubbled through the flask and across two gas scrubbers of 2 M NaOH for 3 hours to quench any remaining phosgene derivatives. THF was removed under reduced pressure and the solution was precipitated in excess ice-cold hexanes. The solid was collected via filtration after cooling for 1 hour and dried in vacuo to yield a slightly yellow solid (1.22 g, 47.2%).

^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 4.22 (s, 2H), 2.86 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6): δ (ppm) = 167.79, 153.04, 51.58, 30.27.

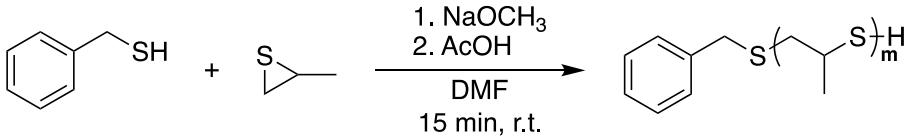
Preparation of FTS-PSarc (**3**)



FTS-Cys (20 mg, 0.077 mmol) was aliquoted into separate vials for each polymer length and suspended in 2mL anhydrous DMF. SarcNCA was added in the corresponding amount for each length (22 units: 132 mg, 1.15 mmol; 29 units: 210 mg, 1.92 mmol) and the vials were place on a horizontal shaker. The FTS-Cys solubilized into solution as the ROP begins, within 30 minutes, and the vials were shaken vigorously for 4 days until gas no longer built up within the container. Polymer samples were precipitated into diethyl ether, collected via filtration, and dried in vacuo to yield a slightly yellow viscous liquid (22 units: 136 mg, 97.1%; 29 units: 175 mg, 97.2%).

^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 7.93 (d, 2H), 7.81 (t, 1H), 7.61 (t, 2H), 4.45 – 3.86 (m, 2H/units), 3.10 – 2.71 (m, 3H/unit).

Preparation of poly(propylene sulfide) (PPS, **4**)

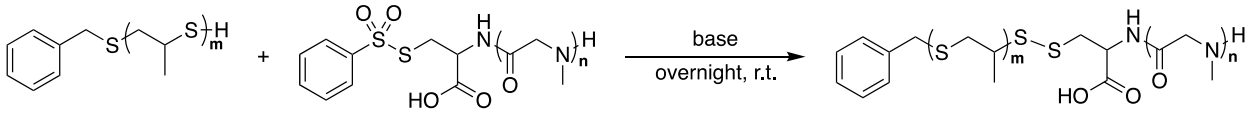


Small molecule-initiated PPS homopolymer polymerization was performed as described previously with slight modifications. [3] Briefly, benzyl mercaptan (13.46 uL, 0.115 mmol) was

added to 1 mL anhydrous DMF and 0.5 M sodium methoxide in methanol (253 uL, 0.123 mmol) in a water bath. After stirring for 5 minutes, propylene sulfide was added in a single rapid injection according to the intended polymer chain length (25 units: 180 uL, 2.3 mmol; 35 units: 288 uL, 3.7 mmol; 62 units: 450 uL, 5.7 mmol). After 15 minutes of polymerization, acetic acid (66 uL, 1.15 mmol) was added to end the reaction. The solution was precipitated in cold methanol, collected, and dried in vacuo to yield a straw-colored viscous liquid (25 units: 155 mg, 69.5%; 35 units: 217 mg, 70.4%; 62 units: 358 mg, 66.5%).

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 7.27 (d, 4H), 2.90 – 2.76 (m, 2H/unit), 2.60 – 2.51 (m, 1H/unit), 1.34 – 1.23 (m, 3H/unit).

Preparation of PSarc-*b*-PPS copolymer (**5**)



The combinatorial library was created using the following general procedure, substituting polymers of varying lengths for each chain. Briefly, the PPS chains were dissolved into a stock anhydrous DMF and aliquoted out into 1.1 eq. portions for each library entry along with 1.1 eq. of base to deprotonate to the thiolate ion. The PSarc chains were dissolved into a stock anhydrous DMF and aliquoted out into 1 eq. portions across the PPS vials. The mixtures were shaken vigorously overnight and precipitated into diethyl ether to yield PSarc-*b*-PPS (36% to 80%).

^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 4.44 – 3.85 (m, 2H/unit PSarc), 2.68 – 2.59 (m, 1H/unit PPS), 1.37 – 1.20 (m, 3H/unit PPS).

b. Nanocarrier assembly

Preparation of nanocarrier formulations via thin film rehydration

Thin film rehydration was performed as described previously.[4] Polymer (5 mg) was dissolved in anhydrous DCM and added to a glass HPLC vial. The solvent was removed via desiccation to leave a thin film along the surface of the glass vial. To this dried film, 0.5 mL milli-Q water or 1X PBS was added and the vial was placed on a horizontal shaker for 18 hours. For loading studies, DiI (0.0625%) or ethyl eosin (0.25%) was added to the DCM/polymer solution before desiccation.

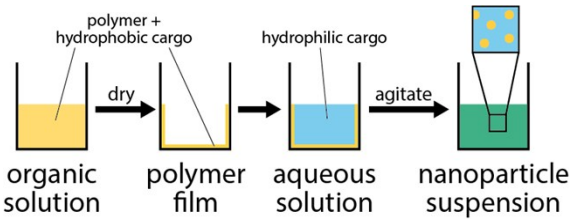


Figure S1. Schematic of thin film rehydration

Preparation of nanocarrier formulations via flash nanoprecipitation

Flash nanoprecipitation was performed using a CIJ mixer following established procedures. [5] Polymer (10 mg) was dissolved in 0.5 mL of THF or DMF and impinged against 0.5 mL milli-Q water or 1X PBS. For loading studies, DiI (0.0625%) or ethyl eosin (EE, 0.25%) was added to the organic layer. A total of 5 impingements was performed, with the final injection into reservoir of 1.5 mL 1X PBS. The resulting mixture was filtered through a Sepharose 6B-CL SEC column to remove the residual organic solvent and unloaded dyes.

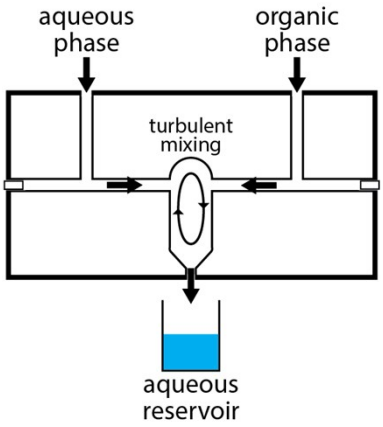


Figure S2. Schematic of flash nanoprecipitation

c. Nanocarrier characterization

Nanocarrier size, polydispersity, and zeta potential characterization

Nanostructure diameter and polydispersity was measured by dynamic light scattering (DLS). Nanostructures were diluted 1:10 in 1x PBS prior to measuring size and PDI. Zeta potential was measured by electrophoretic light scattering (ELS). Nanostructures were diluted 1:10 in ultrapure water prior to ELS. DLS and ELS were performed using a Zetasizer Nano instrument (Malvern Instruments).

Transmission electron microscopy (TEM)

The 1.5% uranyl formate (UF) negative stain was prepared in ultrapure water. 10 N KOH was used to adjust the pH to 4.5. Formvar carbon film copper grids (400 mesh; Electron Microscopy Sciences, Inc.) were glow discharged (25 W, 10 sec). A volume of 3 μl of PSarc-*b*-PPS nanostructure formulation was applied to glow discharged grids. Grids were gently blotted, passaged through two 30 μl volumes of ultrapure water, and were negatively stained by two passages through 30 μl volumes of 1.5% UF stain. Excess stain was removed by gentle blotting. This procedure results in ~0.5 μl of stain on the grid. The activity is 2.55×10^{-5} μCi/grid. A JOEL 1400 Transmission Electron Microscope operating at 120 kV was used to acquire images of each PSarc-*b*-PPS formulation.

Small-angle x-ray scattering (SAXS) data acquisition and analysis

SAXS was performed using synchrotron radiation (10 keV collimated x-rays) at the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) beamline at the Advanced

Photon Source at Argonne National Laboratory. The q-range of 0.001 – 0.5 Angstroms was analyzed. Data reduction, consisting of the removal of solvent/buffer scattering from the acquired sample scattering, was completed using PRIMUS 2.8.2 software while model fitting was completed using SasView 4.0.1 software package. Further data analysis was performed using the core-shell sphere (http://www.sasview.org/docs/user/models/core_shell_sphere.html) and vesicle model fits (http://www.sasview.org/docs/user/models/vesicle.html). The fitting formulae for these models is described below

Core-shell model:

Calculates 1D and 2D scattering as the following:

$$P(q) = \frac{\text{scale}}{V} F^2(q) + \text{background}$$

where

$$F(q) = \frac{3}{V_s}\left[V_c(\rho_c - \rho_s)\frac{\sin(qr_c) - qr_c\cos(qr_c)}{(qr_c)^3} + V_s(\rho_s - \rho_{\text{solv}})\frac{\sin(qr_s) - qr_s\cos(qr_s)}{(qr_s)^3}\right]$$

where

V_s = volume of the whole particle

V_c = volume of the core

r_s (radius + thickness) = radius of the particle, effective radius for S(Q) when P(Q)·S(Q)P(Q)·S(Q)

r_c = radius of the core

ρ_c = scattering length density of the core

ρ_s = scattering length density of the shell

ρ_{solv} = scattering length density of the solvent

Vesicle model:

Calculates 1D and 2D scattering as the following:

$$P(q) = \frac{\phi}{V_{\text{shell}}}\left[\frac{3V_{\text{core}}(\rho_{\text{solvent}} - \rho_{\text{shell}})j_1(qR_{\text{core}})}{qR_{\text{core}}} + \frac{3V_{\text{tot}}(\rho_{\text{shell}} - \rho_{\text{solvent}})j_1(qR_{\text{tot}})}{qR_{\text{tot}}}\right]^2 + \text{background}$$

where

Φ = volume fraction of shell material

V_{shell} = is the volume of the shell

V_{cor} = is the volume of the core

V_{tot} = is the total volume

R_{core} = is the radius of the core

R_{tot} = is the outer radius of the shell, effective radius for S(Q) when P(Q)·S(Q)

$\rho_{solvent}$ = scattering length density of the solvent, same as core

ρ_{scale} = scattering length density of the shell

background = flat background level (incoherent scattering in the case of neutrons)

j_1 = spherical bessel function, $j_1 = (\sin(x) - x\cos(x)) / x^2$

d. In vitro assays

Cell culture

RAW 264.7 cells (murine macrophage cell line) were acquired from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37 °C in the presence of air (95%) and CO_2 (5%).

Cytotoxicity assessment

The viability of RAW 264.7 macrophages in the presence of PSarc-*b*-PPS nanostructures was determined using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. RAW 264.7 cells (3×10^5 cells/mL, 100 µL) were transferred into each well of a 96-well plate and left overnight in the incubator for adherence. The adhered cells were treated with micelle and vesicle nanostructure formulations to attain a final polymeric concentration in each well at 0.125, 0.25, and 0.5 mg/mL and incubated for 24 h. After 24 h, all wells were added with MTT (5 mg/mL in PBS, 10 µL) and further incubated for 4 h. The resultant formazan crystal deposition in each well was dissolved in DMSO (200 µL) and the absorbance was measured at 570 nm. All the samples were analyzed in quadruplicates. The percentage cell viability was calculated as: % cell viability = (OD of treated sample/ OD of untreated sample) × 100.

3. NMR spectra

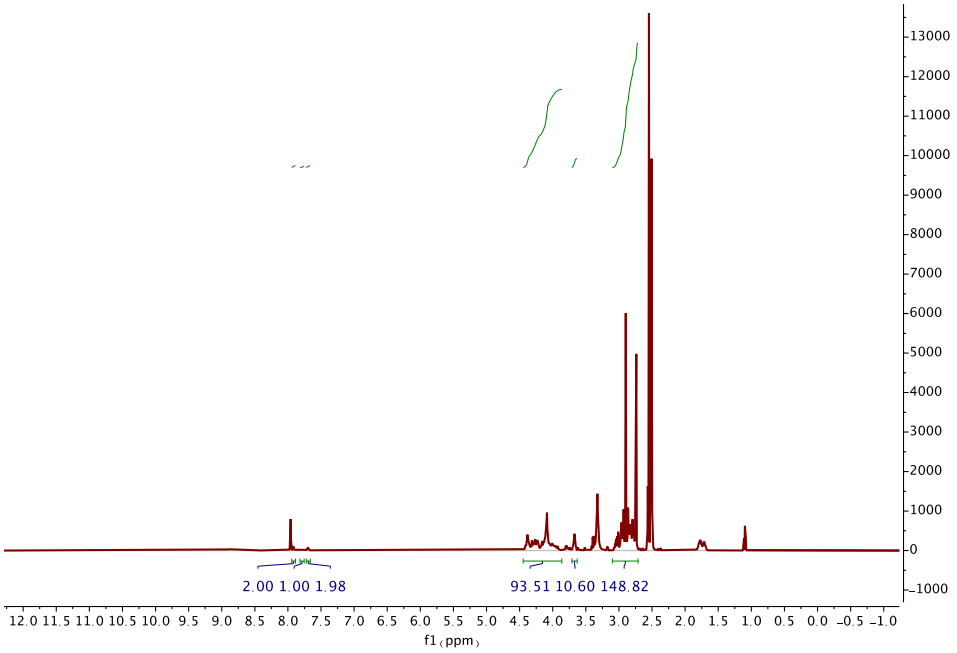


Figure S3. ^{1}H-NMR of FTS-PSarc

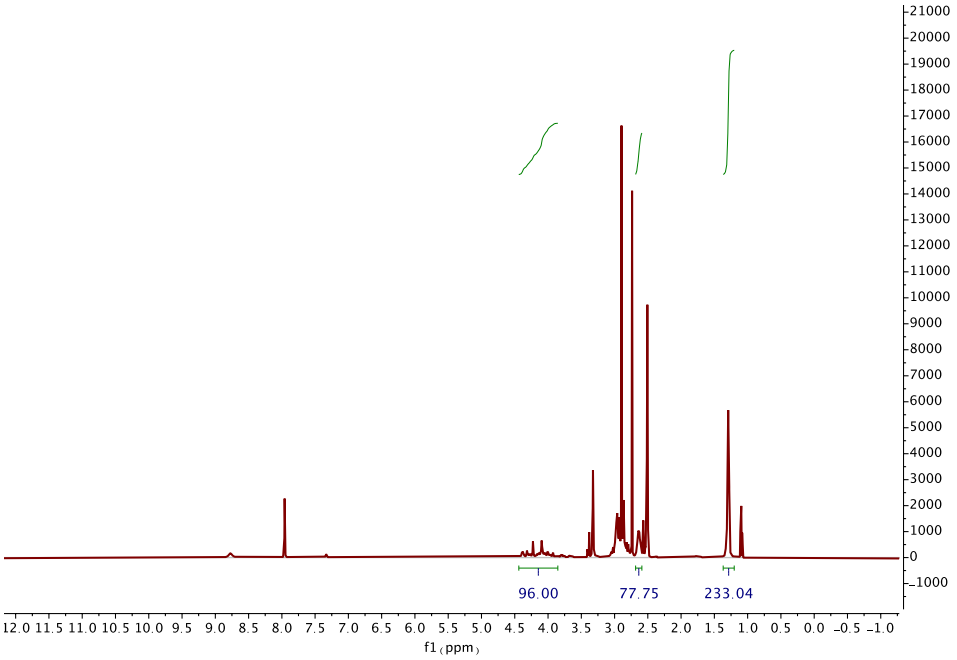
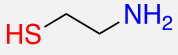
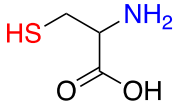
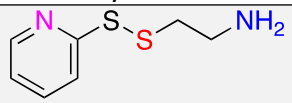
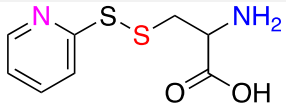
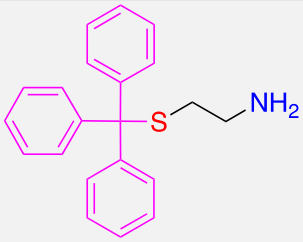
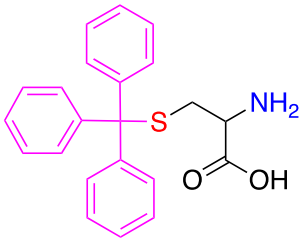
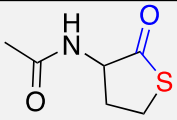
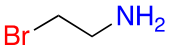
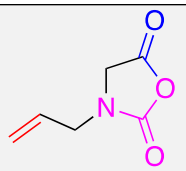
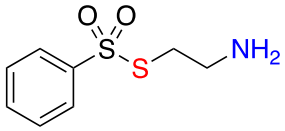
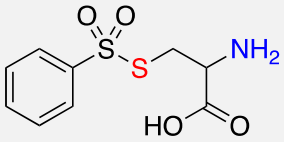


Figure S4. ^{1}H-NMR of PSarc-*b*-PPS

4. Polymer formulation optimizations

a. **Table S1.** Linker selection

	Linker (NCA link in blue PPS link in red)	*Reason for failure (in pink)*
1	 Cysteamine	NCA ring opening with thiol
2	 Cysteine	
3	 Pyr-Cysteamine	NCA ring opening with pyridyl nitrogen
4	 Pyr-Cysteine	
5	 Trt-Cysteamine	PPS instability to deprotection conditions
6	 Trt-Cysteine	
7	 N-Acetylhomocysteine thiolactone	No independent block validation
8	 Bromoethylamine	

9	 N-allyloxycarbonyl glycine NCA	NCA instability
10	 FTS-Cysteamine	Synthetic difficulties
11	 FTS-Cysteine	---

b. **Table S2.** PSarc chains

	[M]/[I]	*DP*	*MW*	*Mass*	*Yield*
1	15	22	1823	136	94%
2	25	29	2320	175	97%
3	40	50	3811	187	84%
4	50	60	4521	227	74%
5	60	80	5941	408	76%

[M]/[I]: monomer/initiator ratio; DP: degree of polymerization; MW: molecular weight

c. **Table S3.** PPS chains

	[M]/[I]	*DP*	*MW*	*Mass*	*Yield*
1	13	14	1127	92	71%
2	20	25	1941	155	70%
3	30	35	2681	217	70%
4	50	62	4679	358	67%

[M]/[I]: monomer/initiator ratio; DP: degree of polymerization; MW: molecular weight

d. **Table S4.** chain cross combinations (red indicates synthesized copolymers)

		PPS			
		14	**25**	**35**	**62**
PSarc	**22**	0.60	0.46	0.38	0.25
	29	0.66	0.52	0.45	0.31
	50	0.77	0.65	0.58	0.44
	60	0.80	0.69	0.62	0.48
	80	0.84	0.75	0.68	0.55

e. **Table S5**. PSarc-*b*-PPS full library

	PSarc	***MW_{PSarc}***	***PPS***	***MW_{PPS}***	***f***	***$MW_{PSarc\text{-}b\text{-}PPS}$***	***Mass (mg)***	***Yield***
1	60	4381	14	1127	0.80	5508	34.8	64%
2	50	3671	14	1127	0.77	4798	33.5	80%
3	80	5801	25	1941	0.75	7742	40.7	74%
4	80	5801	35	2681	0.68	8482	40.5	61%
5	50	3671	25	1941	0.65	5612	31.9	65%
6	50	3671	35	2681	0.58	6352	34.4	62%
7	80	5801	62	4679	0.55	10480	38.1	43%
8	29	2180	25	1941	0.52	4121	60.1	73%
9	22	1683	25	1941	0.46	3624	41.7	58%
10	29	2180	35	2681	0.45	4861	60.4	62%
11	50	3671	62	4679	0.44	8350	31.9	42%
12	22	1683	35	2681	0.38	4364	40.6	47%
13	29	2180	62	4679	0.31	6859	73.4	53%
14	22	1683	62	4679	0.25	6362	46.7	36%

MW: molecular weight; f: hydrophilic weight fraction

f. **Table S6.** PSarc-*b*-PPS thin film rehydration

	PSarc	***PPS***	***f***	***MW***	***Solvent***	***Result***
1	80	62	0.55	10480	DCM/PBS	aggregation
2	22	25	0.46	3624	THF/MQ	aggregation
3	50	62	0.44	8350	DCM/PBS	aggregation
4	29	35	0.45	4861	THF/MQ	aggregation
5	22	35	0.38	4364	THF/MQ	aggregation
6	29	62	0.31	6859	THF/MQ	aggregation
7	22	62	0.25	6362	THF/MQ	aggregation

f: hydrophilic weight fraction; MW: molecular weight

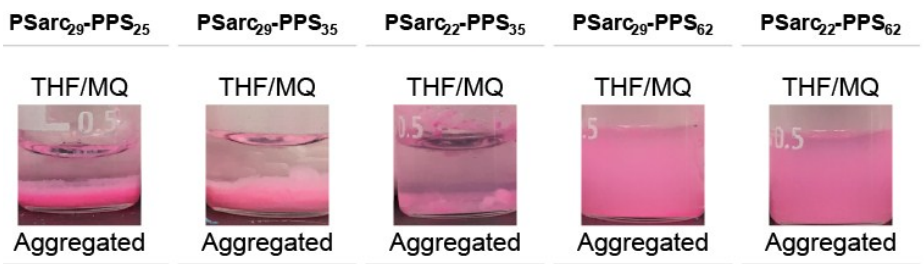


Figure S5. PSarc-*b*-PPS thin film sample results

g. **Table S7**. PSarc-*b*-PPS flash nanoprecipitation

	PSarc	*PPS*	*f*	*MW*	*Solvent*	*d.nm*	*PDI*	*EE%*	*DiI%*
1	50	14	0.77	4798	PBS/DMF	365	0.106	22%	36%
2	50	25	0.66	5612	PBS/DMF	42.9	0.310	49%	42%
3	80	62	0.55	10480	PBS/DMF	78.7	0.560	45%	50%
4	29	25	0.52	4121	MQ/THF	33.5	0.087	---	---
5	29	25	0.52	4121	MQ/DMF	30.5	0.198	---	---
6	22	25	0.46	3624	MQ/THF	276	0.225	---	---
7	50	62	0.44	8350	PBS/DMF	51.7	0.115	45%	31%
8	29	35	0.45	4861	MQ/THF	44.7	0.196	---	---
9	29	35	0.45	4861	MQ/DMF	24.7	0.070	---	---
10	22	35	0.38	4364	MQ/THF	85.9	0.492	---	---
11	29	62	0.31	6859	MQ/THF	80.9	0.767	---	---
12	29	62	0.31	6859	MQ/DMF	74.5	0.395	---	---
13	22	62	0.25	6362	MQ/THF	60.8	0.432	---	---

f: hydrophilic weight fraction; MW: molecular weight; d.nm: hydrodynamic diameter (nm); PDI: polydispersity index; EE%: ethyl eosin encapsulation efficiency; DiI%: DiI encapsulation efficiency

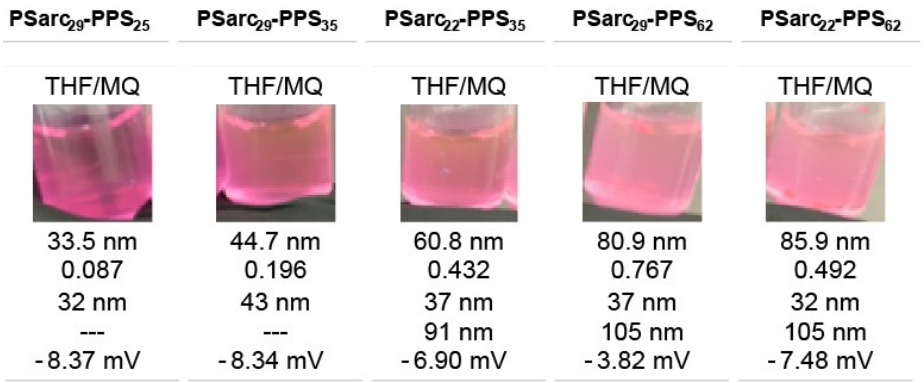


Figure S6. PSarc-*b*-PPS flash nanoprecipitation sample results

5. Cytotoxicity assay

To assess the toxicity of the assembled nanostructures to mammalian cells, we performed an MTT assay using RAW 264.7 macrophages after treatment with copolymer concentrations (0.125 - 0.50 mg/mL). These structures were largely non-toxic, as observed by the cell viability near or above 75% in each case (Fig. S7).

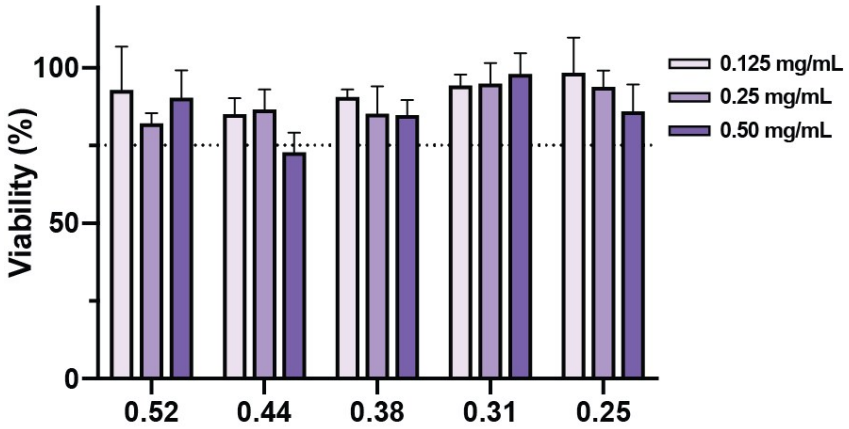


Figure S7. Cytotoxicity of the representative copolymer formulations in RAW 264.7 macrophages dosed from 0.125 mg/mL to 0.500 mg/mL. The dashed line highlights the 75% viability threshold.

6. References

1 D. Huesmann, O. Schäfer, L. Braun, K. Klinker, T. Reuter and M. Barz, *Tetrahedron Letters*, 2016, **57**, 1138–1142.

2 C. Fetsch, A. Grossmann, L. Holz, J. F. Nawroth and R. Luxenhofer, *Macromolecules*, 2011, **44**, 6746–6758.

3 N. B. Karabin, S. Allen, H.-K. Kwon, S. Bobbala, E. Firlar, T. Shokuhfar, K. R. Shull and E. A. Scott, *Nat Commun*, 2018, **9**, 37.

4 S. D. Allen, Y.-G. Liu, T. Kim, S. Bobbala, S. Yi, X. Zhang, J. Choi and E. A. Scott, *Biomater. Sci.*, 2019, **7**, 657–668.

5 S. Allen, M. Vincent and E. Scott, *Journal of Visualized Experiments : JoVE*, 2018.

Contributions. M.A.F. and E.A.S. contributed to the study design. M.A.F. designed the synthetic polymer schemes, conducted all material syntheses with help from R.A.B., assembled nanocarrier formulations, and prepared samples for analysis. M.A.F., M.P.V., and S.B. characterized nanostructure physicochemical properties. M.A.F., M.P.V., and S.B. performed SAXS using synchrotron radiation at Argonne National Laboratory. S.B. modeled SAXS data collected for PSarc-*b*-PPS nanostructures. M.P.V. performed TEM on PSarc-*b*-PPS nanostructures. S.B. maintained in vitro cultures, and S.B. and R.A.B. conducted viability assays.

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