Supporting Information for the Manuscript

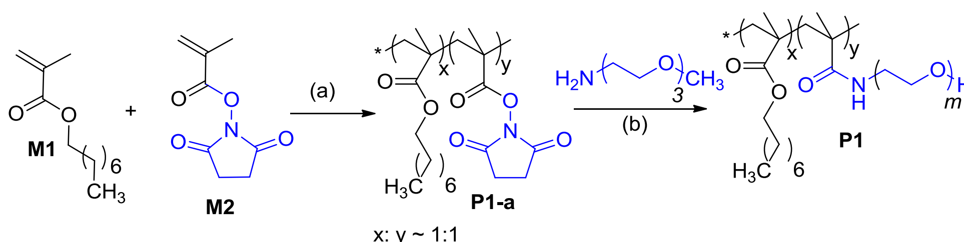
Amphiphilic random copolymer vesicle induces differentiation of mouse C2C12 myoblast

Sumit K Dey,[a, ‡] Krishna Dan,[b,‡] Mahua R Das,[a] Shekhar Saha,[a] Provas Das,[a] Suhrit Ghosh[*b] and Siddhartha S. Jana[*a]

[a] Department of Biological Chemistry, [b] Polymer Science Unit, Indian Association for the Cultivation of Science, 2A & 2B Raja S. C. Mullick Road, Kolkata, India- 700032

‡ Equal contribution by both authors

Materials and methods: Solvents and reagents were purchased from commercial sources and purified by reported methods.[1] ^{1}H NMR spectra were taken from Bruker DPX-500 MHz spectrometer and calibrated using TMS as the internal standard. Fluorescence emission spectral studies were carried out in a FluoroMax-3 spectrophotometer, from Horiba Jobin Yvon. Molecular weight was determined by GPC analysis using Waters GPC system fitted with a Waters 515 HPLC pump and a Waters 2414 Refractive index detector and calibrated with polystyrene as standard.

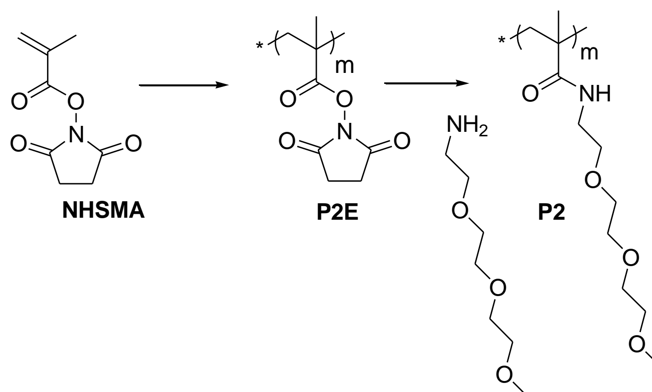


Synthesis of Polymer P1[2]**: P1-a**: Monomer **M1** (0.300 g, 0.0015 mol), **M2** (0.277 g, 0.0015mol), 2-cyano isopropyl di-thiobenzoate (2.0 mg, 0.009 mmol) and AIBN (0.44 mg, 0.0027 mmol) were dissolved in dry degassed anisole (600 μL) in a schlenk flask and the solution was degassed by bubbling Ar for 15 min and sealed and was transferred to a pre-heated oil bath at 95 °C in which it was stirred for 1 h while the reaction mixture solidified. Heating was stopped and the contents were dissolved in 1.0 ml THF and precipitated from excess di-ethyl ether. The precipitate obtained was centrifuged and dried in vacuum to get the desired polymer as white solid (0.350 g, 49 %). M_n=53,000; PDI=1.20, ^{1}HNMR (300MHz, $CDCl_3$, TMS): δ (ppm): 3.97 (broad peak, 2H), 2.78 (broad peak, 2H), 1.62 (broad peak, 2H), 1.21 (broad peak, 10H), 0.88 (broad peak, 3H).

P1: Polymer **P1-a** (0.2g, 0.5 mmol), triethyleneglycolmonomethyletheramine (0.25g, 1.53mmol) and Et_3N (0.105 ml, 0.75 mmol) were dissolved in 0.4 ml dry DMF and the solution was stirred at 80 °C for

12h under N_2 atmosphere. Heating was stopped and the solution was cooled to rt and then dissolved in 6 ml distilled H_2O and purified by centrifugation using excess water in a Amicon ultra-10K (MWCO: 10 KD) centrifugation tube to remove all low molecular weight water soluble compounds. The centrifuged solution was freeze dried to get the pure polymer as an off white sticky compound (0.130 g). ^{1}H NMR (300MHz, $CDCl_3$, TMS): δ (ppm) = 3.92 (broad peak, 2H), 3.64 (broad peak, 12H), 3.38 (broad peak, 3H), 1.29 (broad peak, 3H), 0.89 (broad peak, 3H). M_n=51,000; PDI=1.25.

Synthesis of Polymer P2: Polymer **P2** has been synthesized by a prepolymer based approach as shown below.



Measured amount of Cu(I)Br (2.8 mg, 2 x10^{-5} mole) was taken in a reaction vessel under continuous flow of Ar gas and was added with a solution of PMDETA in degassed anisole (6.9 mg in 0.046 ml). Then 0.2 g (1x10^{-3} mole) N-hydroxy succinimide methacrylate (NHSMA) monomer was added to it followed by 0.15 ml degassed anisole. After that EBIB solution in degassed anisole (3.9 mg in 0.018ml) was added to the reaction mixture and the vessel was sealed and put into a preheated oil bath at 95 °C. Polymerization was continued for 2 h and then the resulting solid was dissolved in 0.5 ml DMF and precipitated out from excess acetone. The precipitate was collected and dried in vacuum to get bluish white solid polymer. Yield = 90 %. M_n= 24000, PDI=1.13, ^{1}H NMR (300 MHz, d_6-DMSO): δ (ppm) = 2.78 (b, 4H), 1.38 (b, 5H).

0.115 g (6.2 x 10^{-4}M) **P2E** and 0.204 g (1.25 x 10^{-3}M) amine of tri-ethylene glycol monomethyl ether was taken in a reaction vessel along with 0.075 g tri-ethylamine in 2.0 ml DMF. Then the reaction vessel was degassed, sealed and was stirred for 12 h at 90 °C. Then the reaction was stopped, cooled to rt and contents were dissolved in 2 ml water and resulting solution was micro-centrifuged in a centrifuged tube (MWCO-3000Da) to remove all water soluble small molecule impurities. Then the solution was freeze dried to get the **P2**. Yield= 66 %. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ (ppm), 3.44 (b, 12h), 1.71 (b, 3H), 1.24 (b, 2H). M_n= 27000, PDI=1.19.

Synthesis of M1: 0.5g ($3x10^{-3}$M) amino tri-ethylene glycol monomethyl ether and 0.624 g ($6.1x10^{-3}$M) tri-ethylamine was dissolved in 2 ml dry dichloromethane and was cooled in an ice bath under Ar atmosphere. To this, 0.36g (4.6×10^{-3}M) acetyl chloride in 3ml dry dichloromethane was added drop-wise at 0°C. After the addition was over the reaction mixture was stirred at rt for 12 h. Then the reaction was stopped and it was washed with saturated brine solution and then the organic part was dried over anhydrous sodium sulphate and concentrated in vacuum to get the pure product as light yellow oil. Yield= 81%, ^{1}H NMR, (300MHz, $CDCl_3$, TMS): δ (ppm), 3.66 (m, 6H), 3.57 (m, 4H), 3.46 (m, 2H), 3.37 (s, 3H), 2.06 (m, 3H).

Solution preparation: Appropriate amount of a given sample (polymer or small molecule) was taken in a vial and added with double distilled water. In case of polymer, repeat unit molecular weight was considered for calculation of concentration. As in the random copolymer (P1) two types of monomers were incorporated in 1:1 ratio, the repeat unit molecular weight = summation of the molecular weight of two types of repeat units with hydrophobic and hydrophilic chains.

Cell culture and myotube formation: Mouse myoblast, C2C12, Chinese hamster ovary, CHO and Mouse neuroblastoma, Neuro-2a cell lines (ATCC, Manassas, VA, USA) were cultured in growth medium, GM [Dulbecco's modified Eagle's medium, DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 units/ ml Penicillin and 100 µg/ ml Streptomycin]. Formation of myotubes was induced by replacing GM with differentiating medium, DM [DMEM containing 2% horse serum, 100 units/ ml Penicillin and 100 µg/ ml Streptomycin]. Images were captured using an Olympus IX-51 microscope at 24 h interval till 96 h.

Cell viability: Cell viability in presence of **P1** was determined using sodium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (Sigma-Aldrich, St Louis, MO, USA) reduction assay according to the previous method.[3] The confluent C2C12, CHO, or Neuro-2a cells were seeded in triplicate in 96 well tissue culture plates (BD Bioscience, CA, USA) at the density of $1x10^4$ cells per well. The cells were allowed to adhere overnight, and then treated with varying concentration 0-500 µM. The untreated cells and media alone were used as positive and negative controls, respectively. The cells were allowed to grow for 24 h in the presence **P1**. After the incubation, GM was replaced with fresh 150 µL medium containing 75µg MTT dye, and plate was incubated at 37^0C for 3h. After removing the dye, 150 µL of DMSO to each well was added and incubated for 1h at room temperature. Absorbance was recorded at 570 nm using a plate reader (Varioskan Flash, Thermo Fisher Scientific Inc., Rockford, IL, USA), and percentage of cell viability was determined following an equation: (%) cell viability = (treated cells- negative control)/(positive control- negative control) x100.

Electronic Supplementary Material (ESI) for Biomaterials Science
This journal is © The Royal Society of Chemistry 2013

Electrophoresis and immunoblot analysis: Cells were briefly washed twice with phosphate buffer saline and directly lysed with Laemmli buffer containing 5% β-mercaptoethanol. Proteins were separated by SDS-PAGE on 8% or 12% polyacrylamide Tris ⁄ glycine gels and transferred to polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA). The membrane was blocked in 5% skim milk with 0.05% Tween-20 in phosphate buffered saline. Then the membrane was incubated overnight at 4 ^{0}C with antibodies against MyoD (1:800), Myogenin (1:800), p21 (1:2000), (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or β-tubulin (1:5000), (Sigma), followed by incubation with horseradish peroxidase conjugated secondary antibodies [anti-rabbit IgG (1:2000) or (anti-mouse IgG (1:5000)] for 2 h at room temperature and developed with Super Signal West Femto reagent (Thermo Fisher Scientific). Relative band intensity was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA) software after normalizing tubulin band intensity.

Immunohistochemistry: Cells were washed with phosphate buffer saline, and fixed with 4% paraformaldehyde solution followed by permeabilization with 0.5% Triton X-100 for 20 mins. Then cells were treated with 0.25% Triton X-100, 2% BSA in 1X PBS for 60 min at room temperature followed by incubation with anti-MHC (1:500) (MF 20, Developmental Studies Hybridoma Bank, University of Iowa, USA) antibody overnight at 4^{0}C. The secondary antibody Alexa 488 goat anti-mouse IgG (1:2000), (Invitrogen) was incubated with cells at room temperature for 1h. Nuclei were counter stained with 4', 6-diamidino-2 phenylindole (DAPI, Sigma) at room temperature for 20 mins. After washing, cover slips were mounted using a Prolong Gold antifade kit (Invirogen). The images were collected using a Nikon C1 confocal microscope (Nikon, Tokyo, Japan).The fusion index was calculated as the number of nuclei in differentiated myotubes divided by the total number of nuclei per field. Myotubes were defined as MF 20 positive multinucleated cells, which contain more than three nuclei.

Lactate dehydrogenase (LDH) assay: Cellular plasma membrane integrity was monitored using the permeability assay based on the release of LDH into the culture medium. This assay is based on the ability of LDH to convert pyruvate to lactate with simultaneous oxidation of NADH (co-factor of LDH) to NAD^{+}. As NAD^{+} has a distinct absorbance at 340 nm, monitoring the absorbance at this wavelength indicates the LDH release. C_2C_{12} cells were seeded in 12 well tissue culture plates (BD Bioscience, CA, USA) at the density of 6.3×10^4 cells per well and were allowed to adhere overnight. 100 µM of **P1** was added to the tissue culture plates and the assay was monitored after 24, 48, 72 and 96 h. For quantification of released LDH in various experiments, cell cultured supernatant from individual experiment was taken out and added to a reaction mixture containing 1.0 mM pyruvate and 0.1 mM NADH in 100 mM sodium phosphate buffer (pH 7.4). Absorbance was recorded at 340 nm on a HITACHI-U 2000 spectrophotometer. As a control experiment, 0.1% triton X-100 solution was added to the cells and the

LDH assay was monitored after 10 min. The percentage of released LDH from the treated cells was calculated by considering triton X-100 treated LDH-release as 100%.

Statistical analysis: Data were expressed as the means ±SEM or means ± SD. Statistical significance was tested with paired sample t-test for means. The differences were considered to be significant if p value was < 0.05.

Additional figures and schemes:

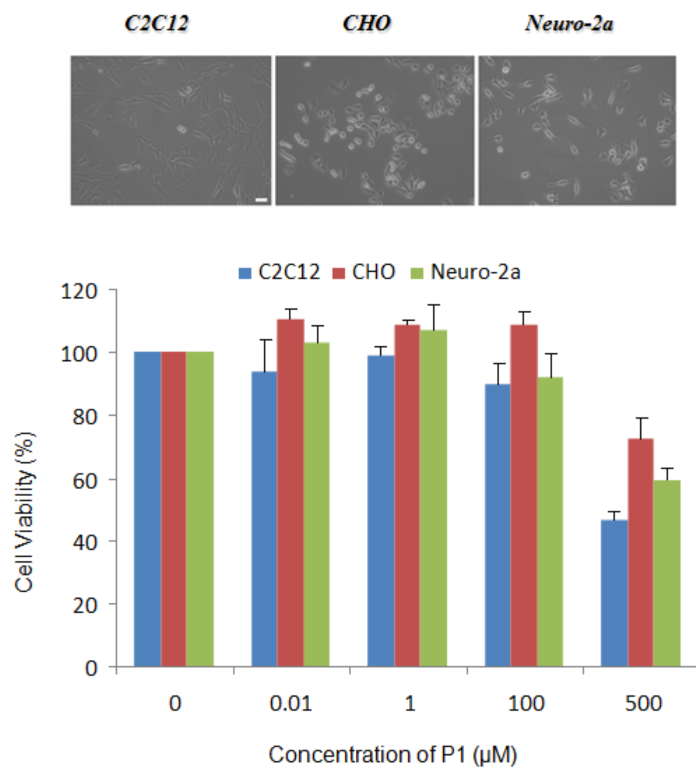


Figure S1: Top- Bright field images of various cells after treating with **P1** (100 μM) for 24 h; Bottom- Cell viability results from MTT assay in presence of **P1** (0-500 μM) for 24 h. Data presented here mean ± SD from three independent experiments. Scale bar = 50 μm.

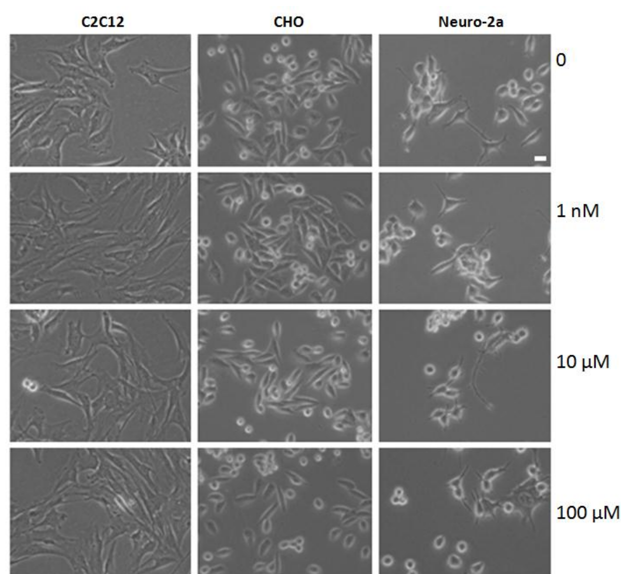


Figure S2: Bright field images of C2C12, CHO and Neuro-2a cells treated with **P1** at various concentrations (as indicated) for 24 h. Scale bar = 50 µm

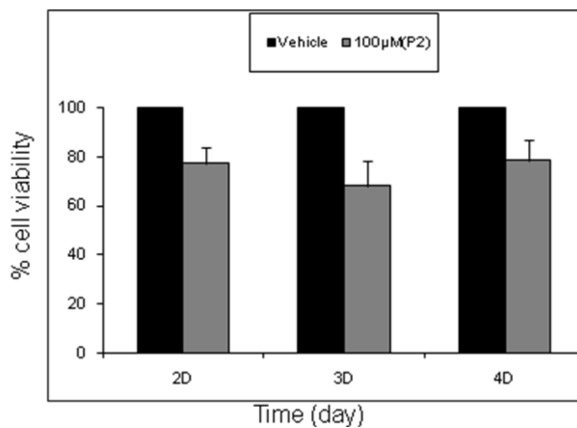


Figure S3: Cell viability results of **P2** (100µM) by MTT assay at different time interval. Controls (vehicle) are 100% cell viable.

Reference:

1. D.D. Perrin, W. L. F. Armarego and D. R. Perrin, *Purification of Laboratory Chemicals, Pergamon Press*: **1986**.

2. K. Dan, N. Bose and S. Ghosh, *Chem. Commun.* 2011, 12491-12493.

3. N. W. Roehm, G. H. Rodgers, S. M. Hatfield and A. L. Glasebrook, *J. lmmunol. Method,* 1991, 257-265