Supporting information for

Hyperbranched vs. Linear Poly(disulfide) for Intra-cellular Drug Delivery

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Materials and methods: Spectroscopic grade solvents, purchased from Spectro Chem Pvt. Ltd, were used for all spectroscopy studies. Synthesis and characterization of L-PDS and HB-PDS have been reported before by our group.[1] Same samples have been used for the present study. All cell lines were purchased from NCCS, Pune, India. UV/ Vis spectra were recorded in a JASCO V-750 spectrometer. TEM images were captured in JEOL-2010EX machine operating at an accelerating voltage of 200 kV. Dynamic light scattering (DLS) measurements were carried out in a Malvern instrument. Fluorescence spectra were recorded in a FluoroMax-3 spectrophotometer. The absorbance of MTT was recorded by the microplate reader (VARIOSKAN, Thermo Fisher). Fluorescence microscopy images were recorded in a confocal laser scanning microscopy (CLSM) in Leica TCS SP8. Flow cytometry data were collected in a BD LSR Fortessa flow cytometer.

Doxorubicin (Dox) encapsulation: 1.2 mg of Dox.HCl in 800 µL THF was added with 40 µL triethylamine-THF solution (stock was prepared by mixing 10 µL triethylamine in 90 µL THF) and stirred for 3 h at rt to get the neutralized Dox. Then 11.0 mg polymer (L-PDS/ HB-PDS) was dissolved in this solution. Next, 3.0 mL milli-Q water was dropwise added to this solution over 1 h time period with constant stirring. The resulting solution was then dialyzed with water for 48 h (dialysis bag MWCO = 3500) to remove THF, triethyl amine hydrochloride salt and non-encapsulated Dox. The resulting solution was then transferred to a vial and the volume was measured to be 4.55 mL and 5.60 mL for HB-PDS and L-PDS, respectively. Accordingly, final polymer concentration was estimated to be 2.4 mg/ mL and 1.9 mg/ mL for HB-PDS and L-PDS, respectively. Dox encapsulation was confirmed from UV/ Vis and fluorescence emission spectra. To quantitatively estimate drug loading content (DLC) and the drug loading efficiency (DLE), firstly the polarity of the core of the aggregates was estimated by encapsulating pyrene probe in L-PDS and HB-PDS micelle in water. From the emission spectra of the pyrene, encapsulated in these aggregates (Figure S1), I_3/ I_1 values were estimated to be 0.94 and 1.1, for L-PDS and HB-

PDS, respectively, as per literature report.[2] Accordingly, molar extinction coefficient of Dox was estimated in 1-propanol and 2-propanol by concentration dependent UV/ Vis spectra (Figure S2). These values were used to estimate the Dox concentration in Dox-L-PDS and Dox-HB-PDS. From these data DLC and DLE were calculated using the following two equations:

DLC = (Weight of encapsulated Dox/ Weight of the polymer) × 100 %

DLE = (Weight of encapsulated Dox/ Weight of Dox in the feed) × 100 %

Calculation of weight fraction of the hydrophobic poly(disulfide) block in L-PDS and HB-PDS: In the original paper in which HB-PDS was reported, [1] it was confirmed that in each polymer on average there were 22 pyridine-disulfide groups which could be quantitatively replaced by thiol-containing oligo-oxyethylene (OE)-chains. In that sense it is a reasonable assumption, that in each HB-PDS, there are 22 OE-chains. Hence the total weight of the OE chains in one polymer = 210 x 22 = 4620. Molecular weight (M_w) of HB-PDS = 17000 g/ mol.[1] Therefore, weight of the hydrophobic PDS segment = 17000- 4620 = 12380. So weight fraction of the hydrophobic segment in HB-PDS = 0.72. Likewise for the linear polymer, molecular weight (M_w) is 13000.[1] Weight of the two PEG chains = 2000 x 2 = 4000. So weight fraction of the PDS block is [(13000-4000)/ 13000] = 0.69.

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) studies: For DLS measurements, the concentration was adjusted to 100 µg/ mL for Dox-HB-PDS and Dox-L-PDS and the measurements were carried out in a Malvern instrument at 25 ^oC. For TEM images, the polymer solution (c = 100 µg/ mL) was drop casted on a 300-mesh carbon coated Cu grid and air dried for 24 h prior to taking images.

Cell culture condition: The human cervical cancer cell line (HeLa) and the human breast cancer cell line (MDA-MB-231) were cultured in a high glucose Dulbecco's Modified Eagle Medium (DMEM) made of 10 % fetal bovine serum and 1 % L-glutamine-penicillin-streptomycin at 37 ^oC in a humidified environment containing 5 % CO_2. Cells were passaged regularly at 80 % confluency to maintain a healthy environment.

Cytotoxicity assay using MTT: To assess cytotoxicity of the Dox encapsulated polymers, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. First, the cells were seeded with density approximately 1×10^4 cells /well in a 96 well plate in presence of

DMEM medium and incubated for 24 h. After that, the medium was replaced by polymer solutions and the cells were incubated for 24 h and 48 h. Next, at each time point 50 µL of freshly prepared MTT solution (5 mg/ mL) in DMEM was added in each well and incubated for 4 h. Then the medium with MTT solution was carefully removed and 100 µL DMSO was added in each well and incubated for 20 min. Then, the plate was gently shaken for 10 min to dissolve all the precipitates in DMSO. The absorbance at 570 nm was recorded by microplate reader (VARIOSKAN, Thermo Fisher). Cell viability was expressed in the ratio of absorbance of cell treated by Dox encapsulated polymers and the absorbance of cell cultured only in media. Experiments were repeated three times and the data have been reported with standard deviation based on the data obtained from three independent experiments. While presenting the data, always Dox concentration in a given Dox-encapsulated polymer sample was estimated using polymer concentration and DLC of that given polymer.

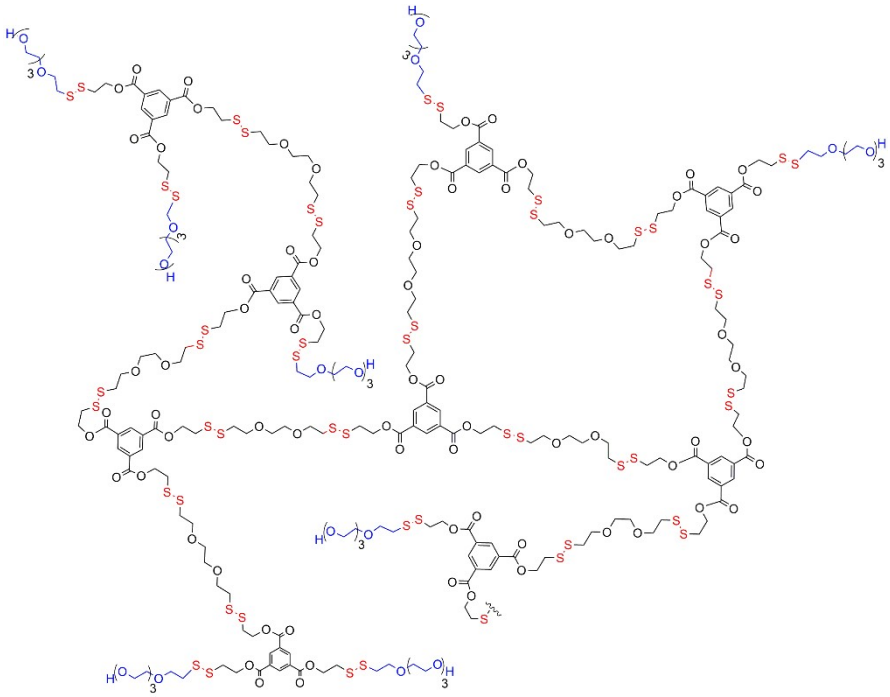
Flow cytometry study: 3×10^5 density of HeLa cell suspension in DMEM was plated in small culture plates and incubated for overnight at CO_2 incubator at 37 ^oC. After cell adhesion, media was replaced by polymer solutions having Dox concentration 10 µg/ mL and incubated for 5 h and 12 h. After each incubation time, the polymer solution was removed, and cells were washed with DMEM several times. Then cells were detached and transferred to DMEM in a FACS tube. Experiment was performed in a BD LSR Fortessa flow cytometer.

Confocal microscopy imaging for intracellular drug delivery: 200 µL of HeLa cells suspension in DMEM (cell density = 1×10^5) was plated in confocal imaging plate and incubated for overnight at 37 ^oC in CO_2 incubator. Next day, the DMEM media was removed and washed with PBS buffer. Then cells were allowed to incubate with Dox encapsulated polymer for 5 h and 12 h at 37 ^oC. After the desired incubation time, Dox encapsulated polymer solution was removed and washed with PBS solution for several times. Then cells were stained with Hoechst 33342 dye for 15 min and washed again with PBS buffer for three times. After that, the cell was immersed in DMEM media and live imaging was done in confocal microscopy.

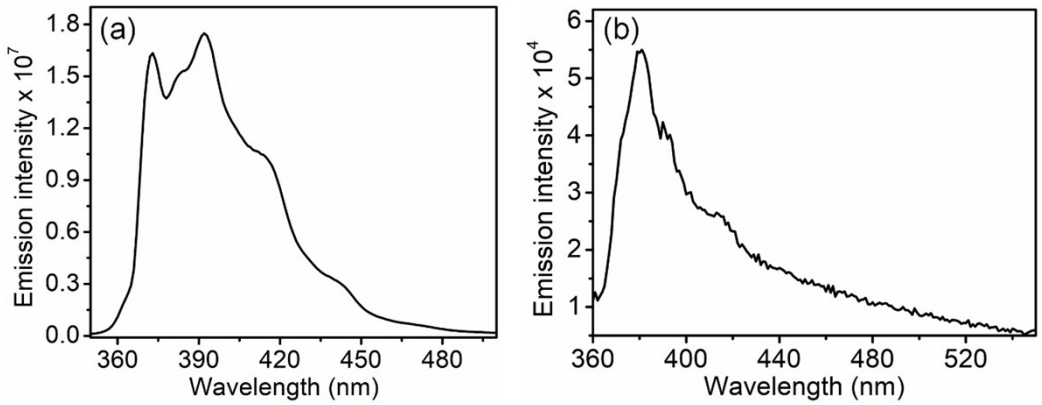
Glutathione induced drug release: 500 µL of Dox encapsulated polymer solution (polymer concentration = 0.5 mg/ mL) was taken in dialysis bag (MWCO = 3.5k Da) and it was dipped in 25 mL of 20 mM aqueous GSH solution. After certain time-intervals, fluorescence emission intensity of the dialysate solution was checked to estimate the amount of drug (Dox) released from

the dialysis bag. This process was continued until the emission intensity reached to a near constant value. Normalized emission intensity in each experiment was plotted as a function of time and from this plot the approximate time for drug release was estimated. In case of Dox-HB-PDS, visible precipitation was noticed in the inner surface of the dialysis bag. As we have used the neutralized Dox, hence its solubility is limited in buffer. Hence the drug release kinetics shown in Figure 1 may be considered in qualitative terms.

Uptake kinetics in presence of DTNB: Cancer cells (HeLa) were plated with cell density ~ 2×10^5 in culture dish and incubated to adhere for overnight in 37 ^oC at CO_2 incubator. Next day, the media was replaced with the solution of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) in DMEM (*c* = 1.2 mM) and incubation was continued for 30 min. Then cells were washed with DMEM 2-3 times. Next, the Dox encapsulated polymer solution (concentration of Dox = 10 µg/ mL) was added and cells were incubated for 4 h. After that, cells were treated with trypsin EDTA to detach them and then DMEM media was added to collect the cell pellets by centrifugation. After removing the supernatant, cell pellets were resuspended in DMEM and the absorbance was measured at 480 nm using microplate reader.



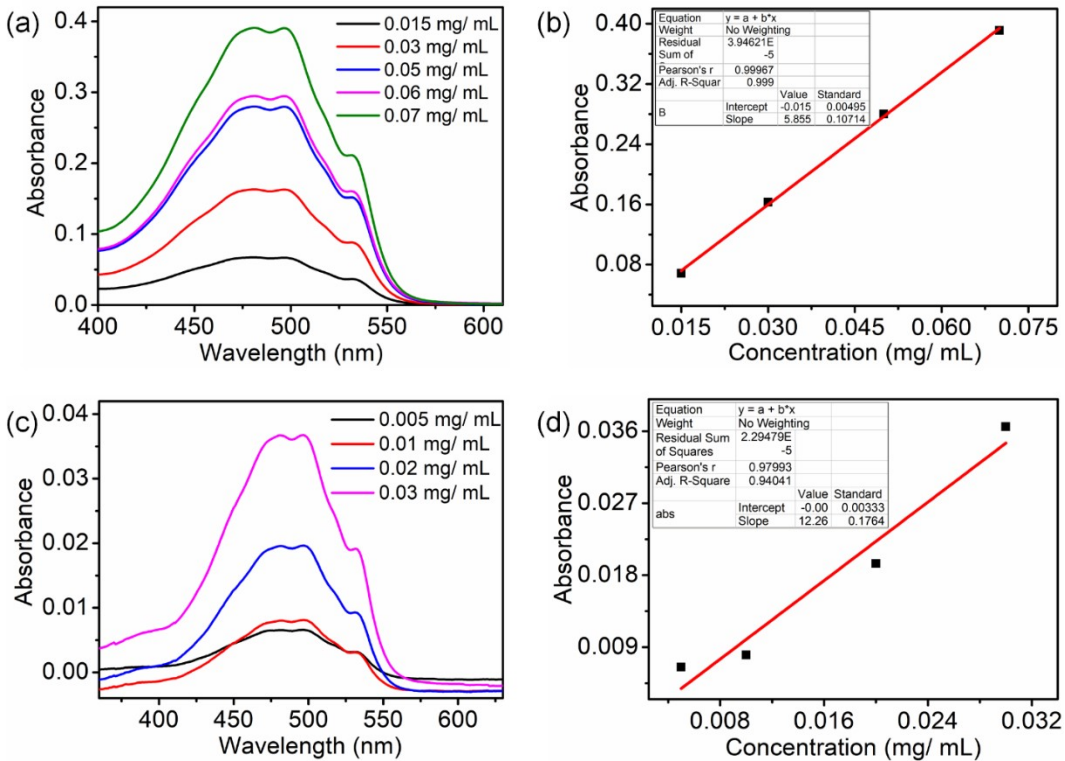
Scheme S1: A representative structure of HB-PDS. This structure does not represent actual number of dendritic/ linear/ terminal units. This is shown just to provide a better clarity about the hyperbranched polymer structure.



Additional Figures:

Fig S1. Emission spectra of pyrene encapsulated aqueous solution of (a) L-PDS and (b) HB-PDS. Polymer conentration = 20 μM, λ_{ex} = 337 nm. I_3 (383 nm)/ I_1 (372) was calculated to be 0.94 and 1.1 for L-PDS and HB-PDS, respectively, from these two spectra. These values were matched with literature[2] to conclude the polarity of the hydrophobic domain is similar to 1-propanol and 2-propanol for the aggregates of L-PDS and HB-PDS, resepctively.

Fig S2. Concentration dependent UV/ Vis spectra of Doxorubicin in (a) 1-propanol and (c) 2-propanol. l = 0.1 cm; (b, d) Absorbance (λ = 482 nm) vs. concentration plot from the data shown in (a) and (c), respectively.



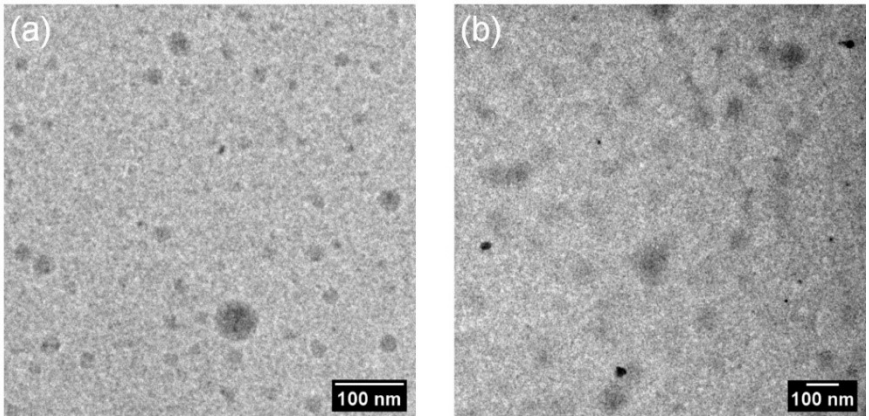
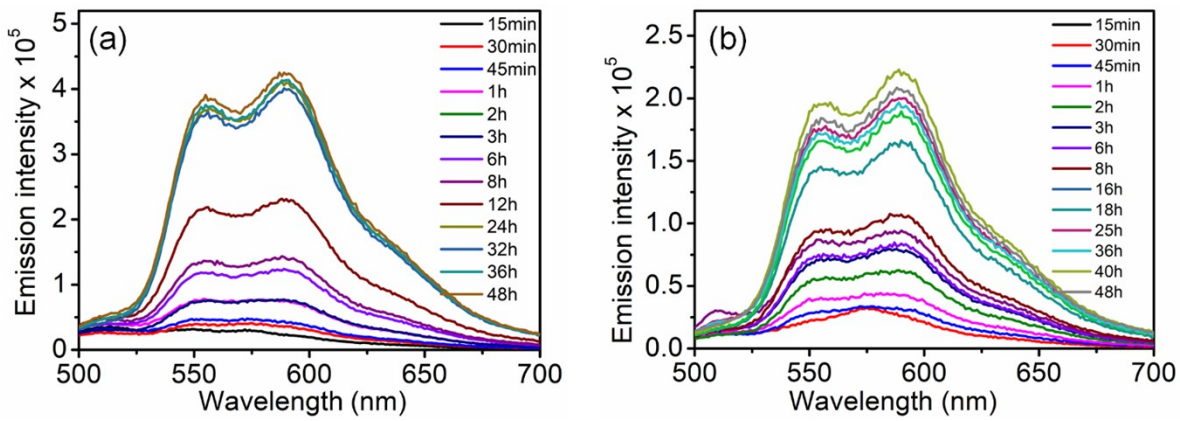


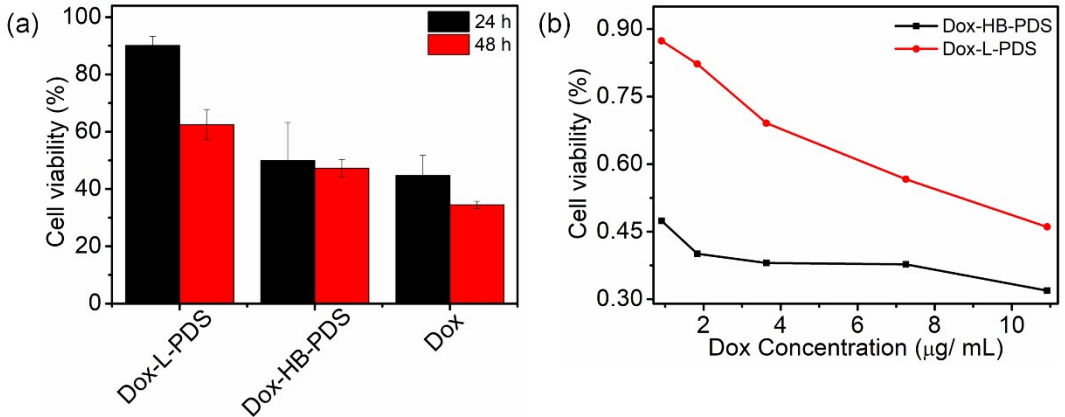
Fig S3. TEM images of Dox encapsulated aqueous solution of (a) L-PDS and (b) HB-PDS



(Polymer concentration = 100 µg/ mL).

Fig S4. Emission spectra of released Dox in presence of 20 mM GSH in PBS buffer (pH = 7.4) at different time point for (a) Dox-HB-PDS and (b) Dox-L-PDS. Polymer concentration = 500 µg/ mL, λ_{ex} = 480 nm.

Fig S5. (a) Cell viability (estimated by the MTT assay) of HeLa cells (at 24 h and 48 h) in presence of free Dox, Dox-HB-PDS and Dox-L-PDS. In each experiment Dox concentration is 10 µg/ mL;



(b) Dox-concentration dependent cell viability of HeLa cells after 24 h incubation in presence of Dox-HB-PDS and Dox-L-PDS.

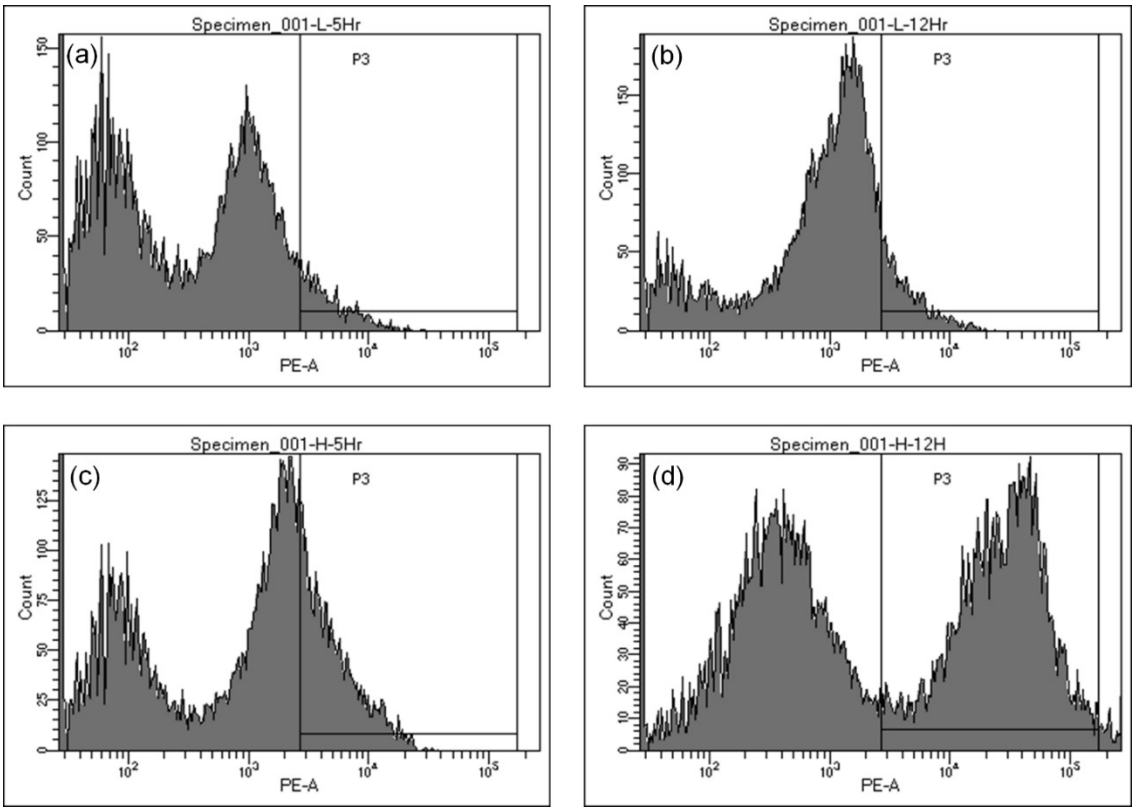


Fig S6. Flow cytometry histogram of HeLa cells treated with (a, b) Dox- L-PDS or (c, d) Dox-HB-PDS after (a, c) 5 h and (b, d) 12 h.

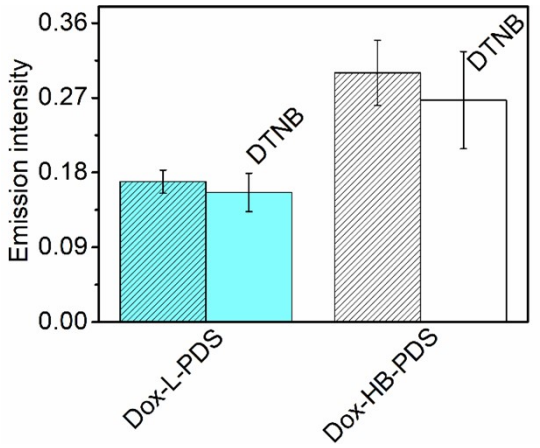


Fig S7. Cellular uptake of Dox-L-PDS and Dox-HB-PDS in presence of DTNB (c = 1.2 mM) after 4 h incubation with HeLa cells.

Reference

1. R. Bej, P. Rajdev, R. Barman and S. Ghosh, *Polym. Chem.,* 2020, **11**, 990.

2. K. Kalyanasundaram and J. K. Thomas, J. *Am. Chem. Soc.,* 1977, **99**, 2039.