Targeting CD4+ Cells with Anti-CD4 Conjugated Mertansine Loaded Nanogels

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Materials:

Polyethylene glycol monomethyl ether acrylate (PEGMA; 480), 2,2'-dipyridyldisulfide, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid, DL-dithiothreitol (DTT), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) were obtained from Sigma-Aldrich. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was purchased from Sigma-Aldrich and purified by recrystallization in cold methanol for three times. DMEM, RPMI-1640, sodium pyruvate, penicillin/streptomycin and L-glutamine were obtained from HyCloneTM (Catalog Numbers: SH30243, SH30255, SH30239, SV30010 and SH30034, respectively). Fetal bovine serum (FBS) was bought from Peak Serum (Catalog Number: PS-FB3).

Methods:

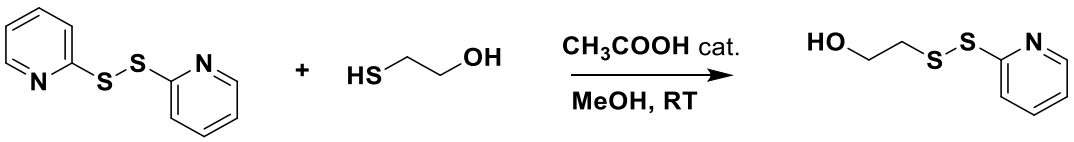
CD4 Cell ELISA

For each well of 96-well ELISA plate, 100,000 $CD4^{high}$ mT-ALL cells were dispersed in 100 μL of PBS. The plate was centrifuged at 500xg for 10 minutes at room temperature. 100 μL of 8% paraformaldehyde was added to each well to fix the cells. Immediately after the addition of paraformaldehyde, plate was centrifuged again for 10 minutes. The plate was incubated at room temperature for additional 15 minutes before paraformaldehyde solution was carefully aspirated from each well. Fixed cells were washed 3 times with 300 μL of PBS and incubated in 200 μL/well of 2X Blocking Solution for 2 hours at room temperature. After this blocking step, 100 μL/well of 1X Incubation Buffer containing titrated antibody (anti-CD4 for standard) was added to each well and the plate was incubated overnight at 4°C. After incubation with anti-CD4 containing buffer, plate was washed 3 times with 300 μL of PBS. Secondary antibody of anti-Rat IgG-HRP (Abcam,

Catalog Number: ab97057) was diluted 1000 times in 1X incubation buffer. 100 μL of prepared secondary was added to each well and the plate was incubated at room temperature for 2 hours. Afterwards, the plate was washed with PBS buffer three times. Each well was incubated with 100 μL of ELISA substrate solution. When blue color developed across the standards, 50 μL of Stop Solution (2N Sulfuric Acid) was added to each well to stop the reaction. The plate was then read at 450 nm. ELISA plate, blocking solution and Incubation buffer were purchased from Abcam and are a part of In-Cell ELISA (ICE) Support Pack (Catalog Number: ab111542).

Synthesis of PDSMA monomer

The monomer was synthesized using method listed in previous reports.[1,2]



Step 1: Briefly stated, 2,2'-dipyridyldisulfide (90.8 mmol, 1 eq) was dissolved in methanol, a few drops of acetic acid were added and stirred for 10 minutes. Following this, 2-mercaptoethanol (56.75 mmol, 0.625 eq) was added dropwise and the reaction stirred at room temperature for 12 hours. The solvent was evaporated to afford a yellow oil. The by-product pyridinthione was recrystallized by placing the reaction mixture in 1:1 hexane ethyl acetate mixture at -20 $^{\circ}$C overnight. The crystals were filtered, and the reaction mixture was purified using column chromatography carried out in hexane/ethyl acetate. The product is a pale-yellow oil. The product yield was 51%. The purity of the compound was checked by ^{1}H-NMR (Figure S1). ^{1}H-NMR (400 MHz, $CDCl_3$): 8.35 (m, 1H), 7.48 (m, 1H), 7.36 (m, 1H), 7.02 (m, 1H), 5.60 (m,1H), 3.70 (t, 2H), 2.83 (m, 2H).

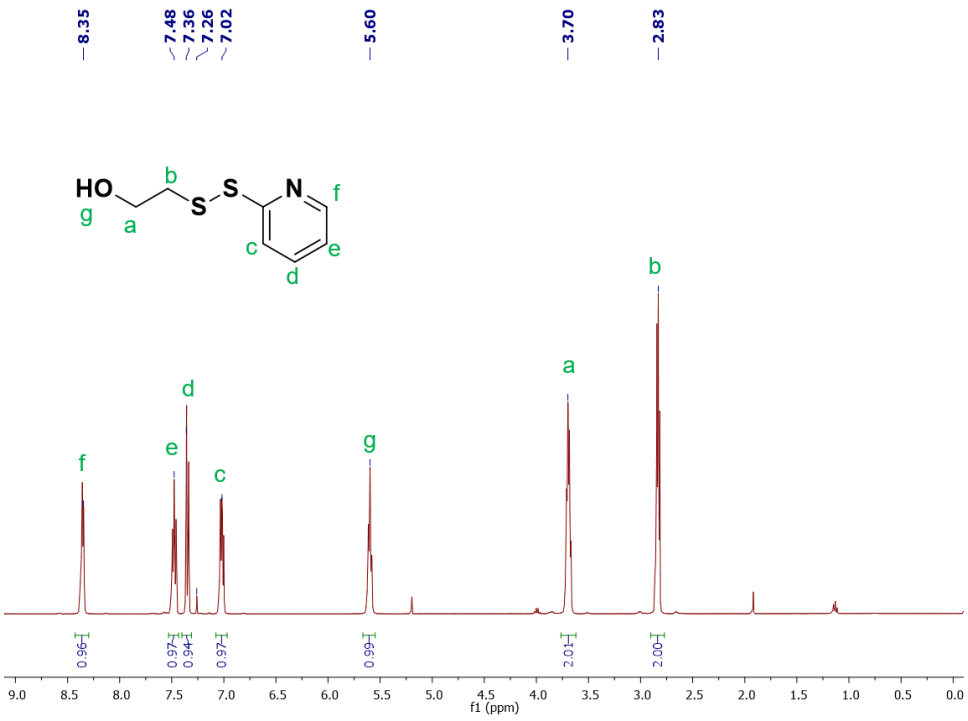
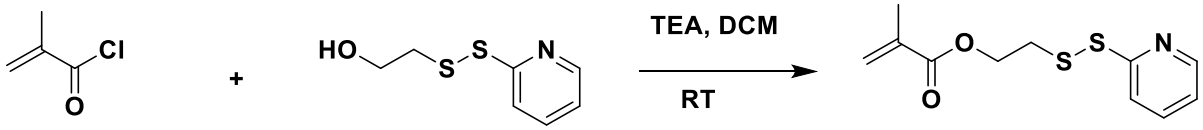


Figure S1: ^{1}H-NMR spectra of 2- (pyridine-2-yldisulfanyl) ethanol



Step 2: The product obtained in step 1 (22.9 mmol, 1 eq) was dissolved in dichloromethane and triethylamine (1.5 eq) was added under inert conditions. The reaction mixture was placed in an ice bath and methacryloyl chloride (25.2 mmol, 1.1 eq) was dropwise added. The reaction was stirred in ice, slowly warmed up to room temperature and stirred for 3 hours. The reaction mixture was extracted with saturated sodium bicarbonate solution followed by washes with brine. Dichloromethane was evaporated and the reaction mixture was purified over silica column chromatography using hexane/ethyl acetate as eluents. The product pyridyl disulfide ethyl methacrylate was obtained as yellow liquid. The product yield was 85%. ^{1}H-NMR (400 MHz,

$CDCl_3$) : 8.46 (m, 1H), 7.67 (m, 2H), 7.08 (m, 1H), 6.12 (d, 1H), 5.57 (d, 1H), 4.39 (t, 2H), 3.08 (t, 2H), 1.93 (s, 3H) – Figure S2.

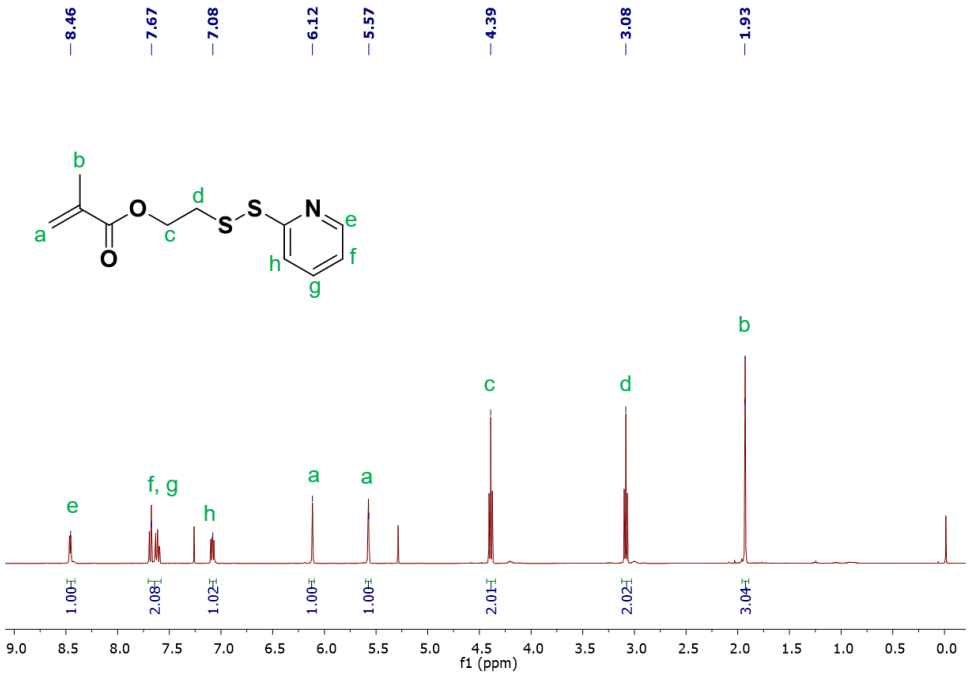
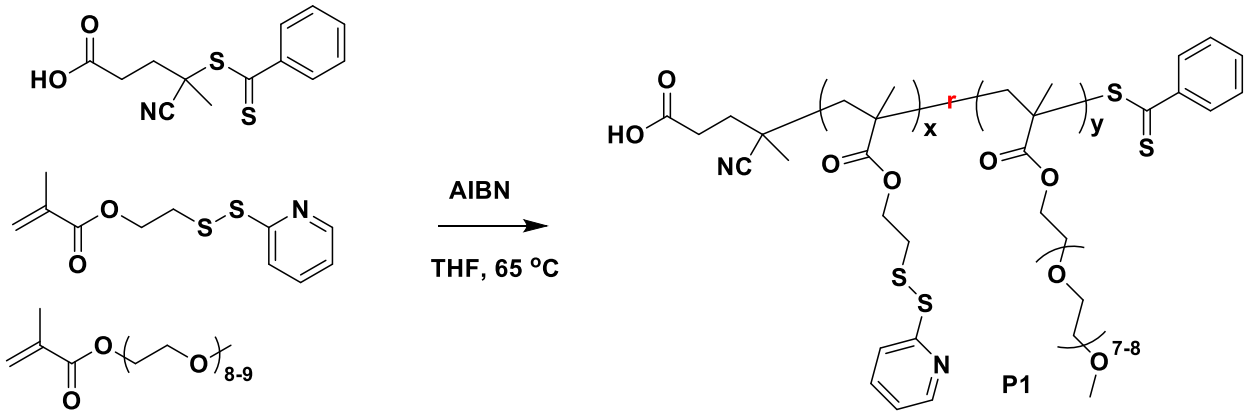


Figure S2: ^{1}H-NMR spectra of pyridyl disulfide ethyl methacrylate (PSDMA)

Synthesis of p(PDS-co-PEG)



PEGMA (1.28 g, 2.56 mmol), PDSMA (1.53 g, 5.98 mmol), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (27 mg, 0.0095 mmol) and AIBN (3.1 mg, 0.0019 mmol) was weighed in a 10 mL Schlenk flask. 3 mL dry degassed tetrahydrofuran was added to the flask and sealed. Three freeze-pump-thaw cycles were performed to degas the reaction mixture. The flask was placed in a pre-heated oil bath maintained at 65 °C and stirred for 24 hours. The polymerization was quenched by dipping the flask in liquid nitrogen and exposing to air. The viscous-pink polymer P1 (2.3 g, yield = 82%) formed was purified by dialysis in dichloromethane/methanol (1:1) and dried with high vacuum. GPC (THF): M_n 22.4 kDa. PDI: 1.26 ^{1}H-NMR (400 MHz, $CDCl_3$): 8.45, 7.66, 7.10, 4.21, 4.06, 3.63, 3.37, 3.02, 1.71, 0.88 (Figure S3). From the NMR analysis, the methoxy peak from PEG (δ 3.37) and aromatic peaks from PDS were used to calculate the ratio of monomers in the polymer. [PDS]/[PEG] x:y = 0.69:0.31. ^{13}C NMR (CDC_{13}) is shown in Figure S4 and the GPC trace is shown in Figure S5.

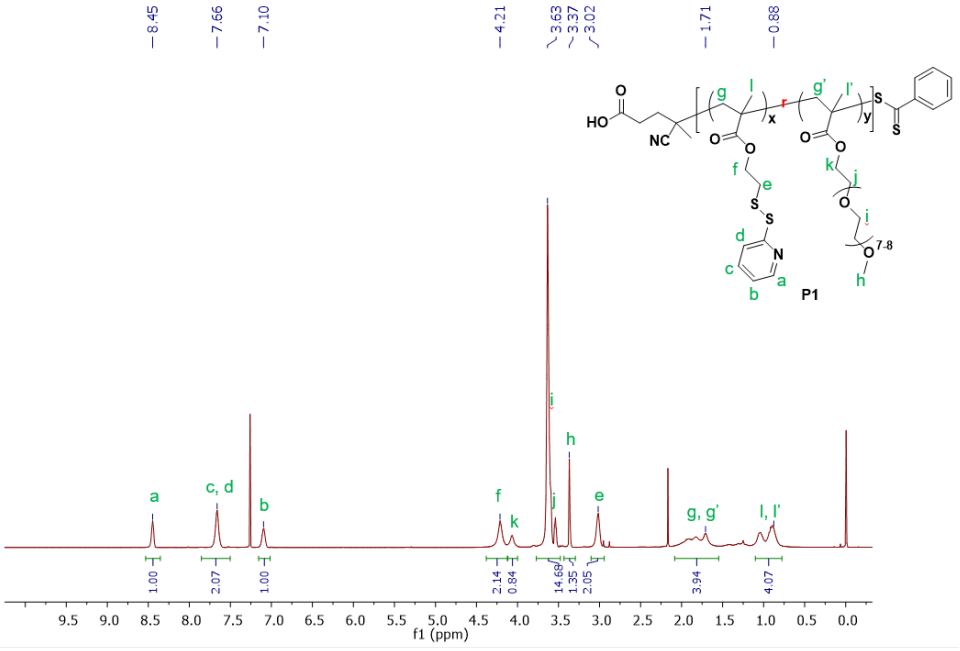


Figure S3: ^{1}H-NMR spectra of polymer - p(PEGMA-co-PDSMA)

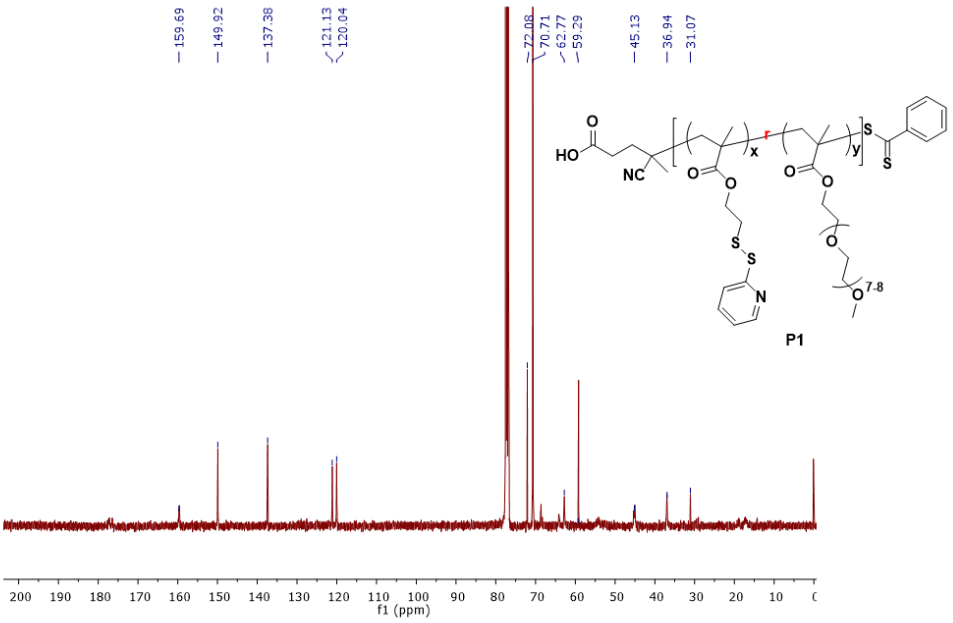


Figure S4: ^{13}C-NMR spectra of polymer - p(PEGMA-co-PDSMA)

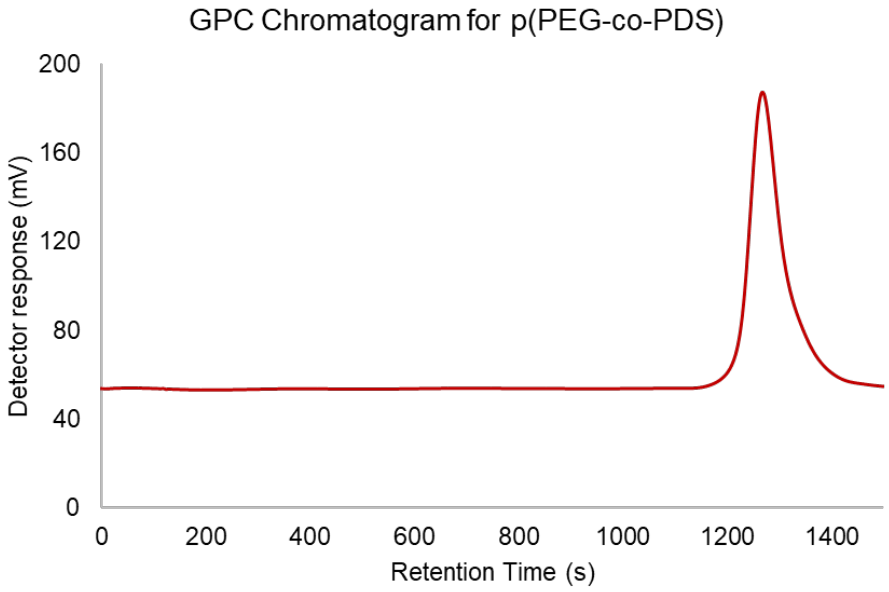


Figure S5: Gel Permeation Chromatograph for p(PEGMA-co-PDSMA)

Synthesis of mertansine (DM1) conjugated polymer

The anti-cancer drug mertansine (DM1) conjugated polymer was made by post-polymerization modification of P1. A 10 mg/mL solution of polymer P1 in distilled water was made and mixed with DM1 dissolved in DMSO. A 100 mM stock solution of DM1 in DMSO was made. An equivalent of 10% of the PDS units was mixed with polymer solution and stirred in inert conditions. % DM1 conjugation was estimated by quantifying the amount of DM1 released. The solution was lyophilized and re-dissolved in $CDCl_3$ for ^{1}H-NMR and ^{13}C-NMR. From the ^{1}H-NMR (Figure S6), the small peaks at chemical shifts of 6.83, 6.41, 5.65, 5.42, 4.74 were attributed to conjugated mertansine as compared with the NMR of mertansine from commercial source MedChemExpress. The ^{13}C-NMR of DM1 conjugated polymer is shown in Figure S7.

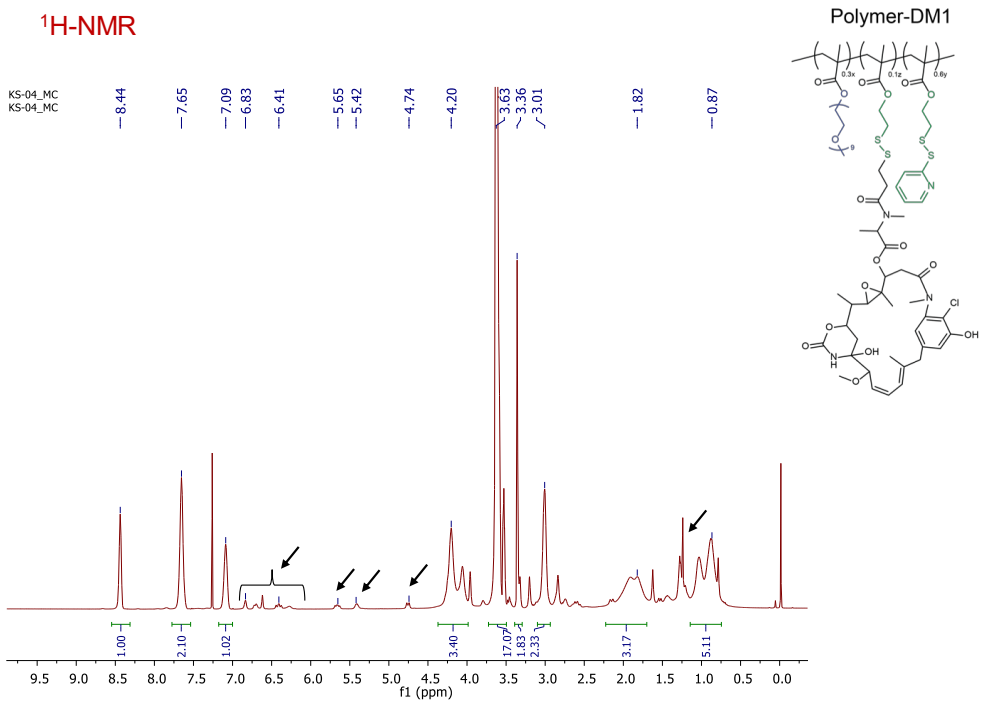


Figure S6: ^{1}H-NMR spectra for mertansine conjugated polymer. The peaks belonging to the mertansine molecule are shown with an arrow.

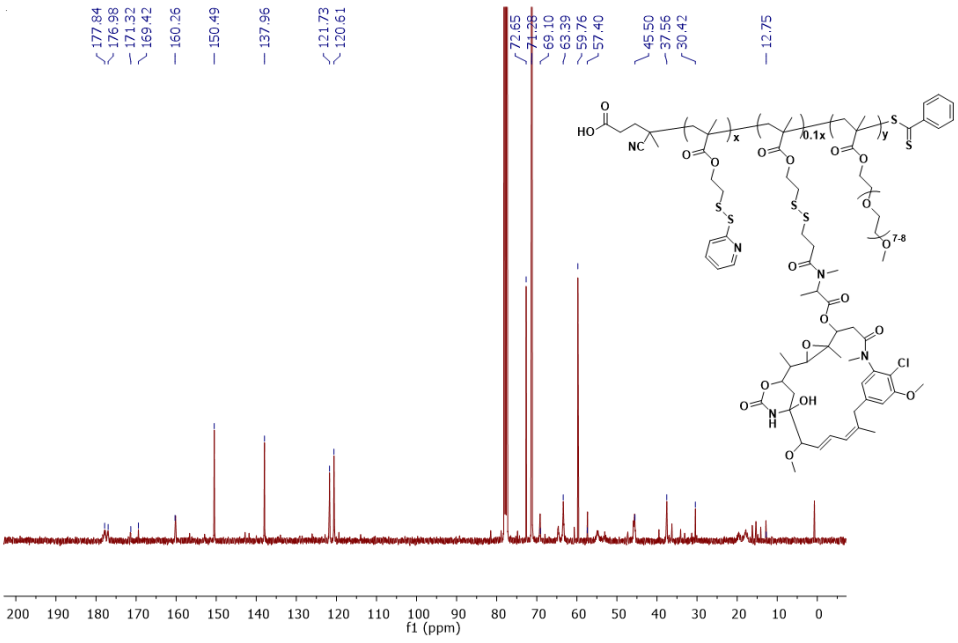


Figure S7: ^{13}C-NMR spectra for mertansine conjugated polymer

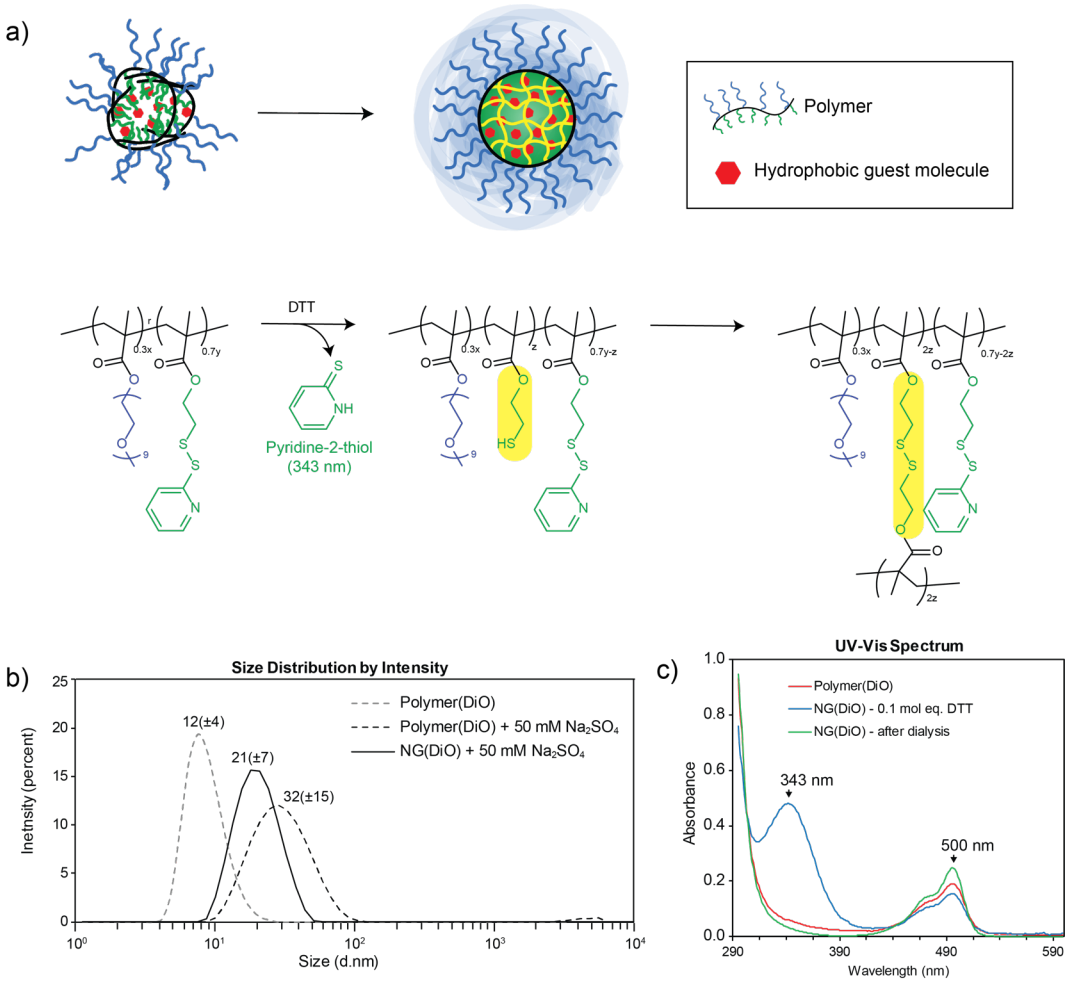


Figure S8: a) Schematic representation of guest molecule encapsulation (upper panel), structure of the polymer and NG (lower panel). b) Dynamic light scattering results of polymer and NG. In the presence of 50 mM Na_2SO_4 aggregate size is increased to desired range. c) UV-vis spectrum of DiO encapsulated polymer and NG. The peak at 343 nm is due to the released pyridine-2-thiol groups. DiO absorbance can be seen at 500 nm.

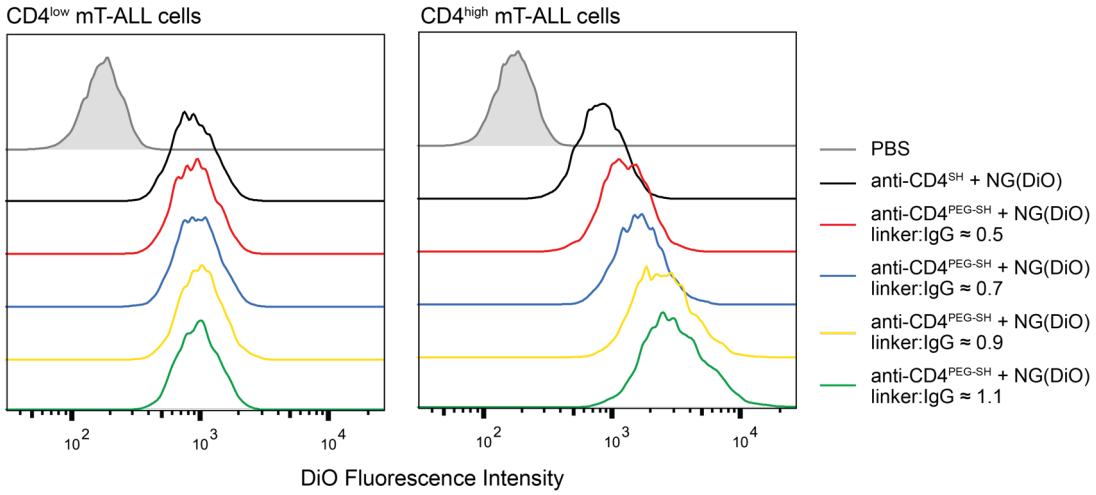


Figure S9: Native anti-CD4 and anti-CD4$^{PEG\text{-}PDS}$ with different linker ratio were treated with 2.5 molar excess TCEP for 2 minutes, then mixed with NG(DiO) to allow conjugation. Resulting products were incubated with co-culture of mT-ALL cells at 37 °C for 3 hours and subsequently analyzed on flow cytometry.

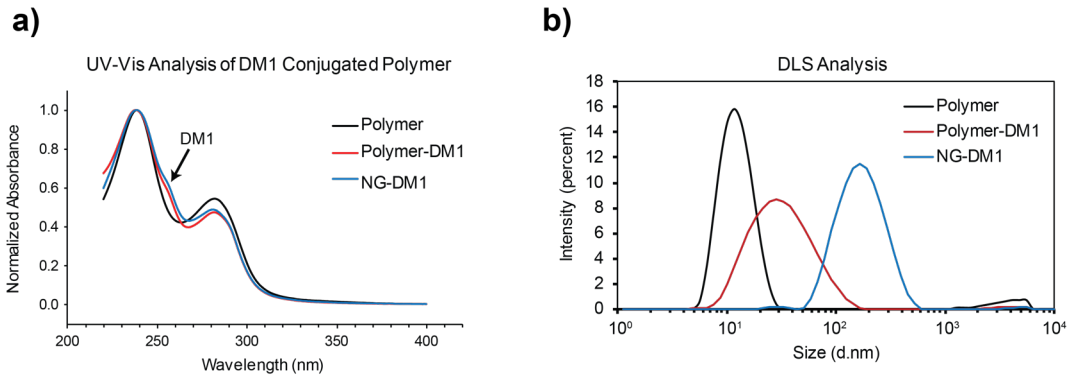


Figure S10: a) UV-vis spectrum of polymer, polymer-DM1 and NG-DM1. DM1 peak is visible at 252 nm. b) Dynamic light scattering results of polymer, polymer-DM1 and NG-DM1.

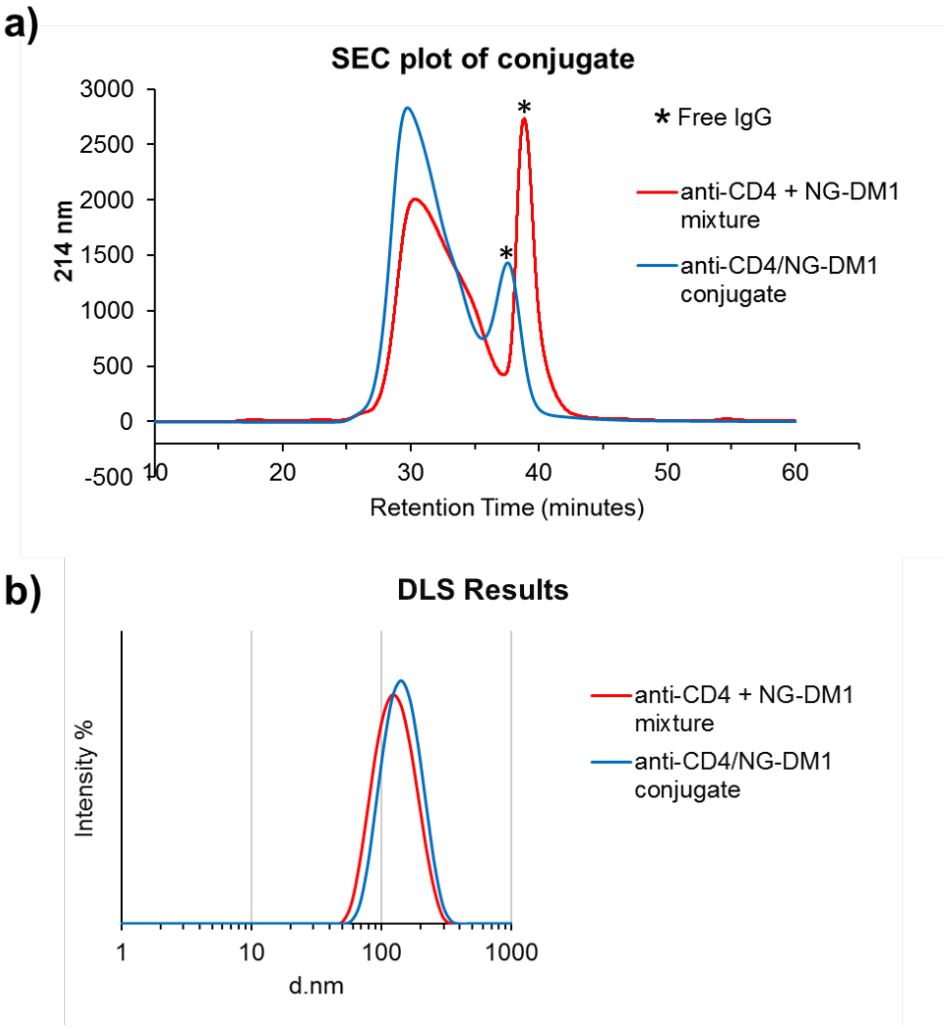
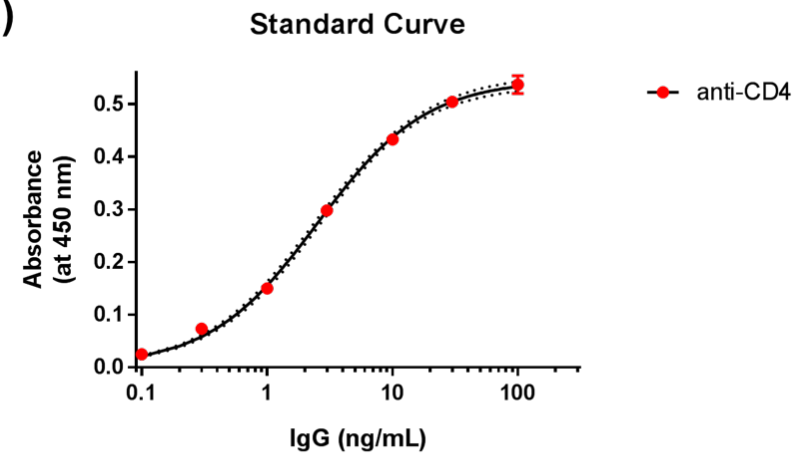


Figure S11: a) SEC analysis of anti-CD4 + NG-DM1 mixture and anti-CD4^{PEG}/NG-DM1 conjugate. The samples were run through Superdex 200 Increase (GE) column in PBS buffer with 0.5 mL/min flow rate. The wavelength detector was set at 214nm. The peak at 40 minutes belongs to the free antibody in the solution. b) DLS analysis of SEC purified NG-DM1.

a)



	Dilution factor	Absorbance (at 450 nm)	Concentration	Concentration * Dil. Factor	Average
anti-CD4^{PEG}/NG-DM1 (125 µg/mL of NG)	500	0.336	3.998 ng/mL	1.999 µg/mL	1.945 µg/mL
	1000	0.235	1.891 ng/mL	1.891 µg/mL	

b)

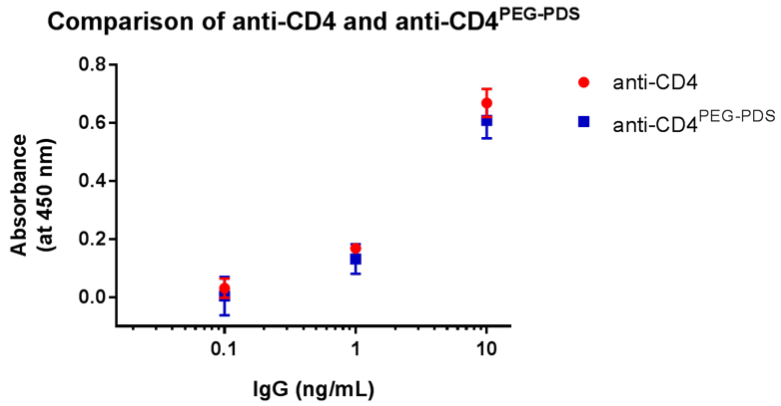


Figure S12: a) ELISA standard curve of anti-CD4 antibody. The graph shows the calculation of anti-CD4 amount conjugated to NG-DM1. b) comparison of native and PEGylated forms of anti-CD4 on ELISA assay.

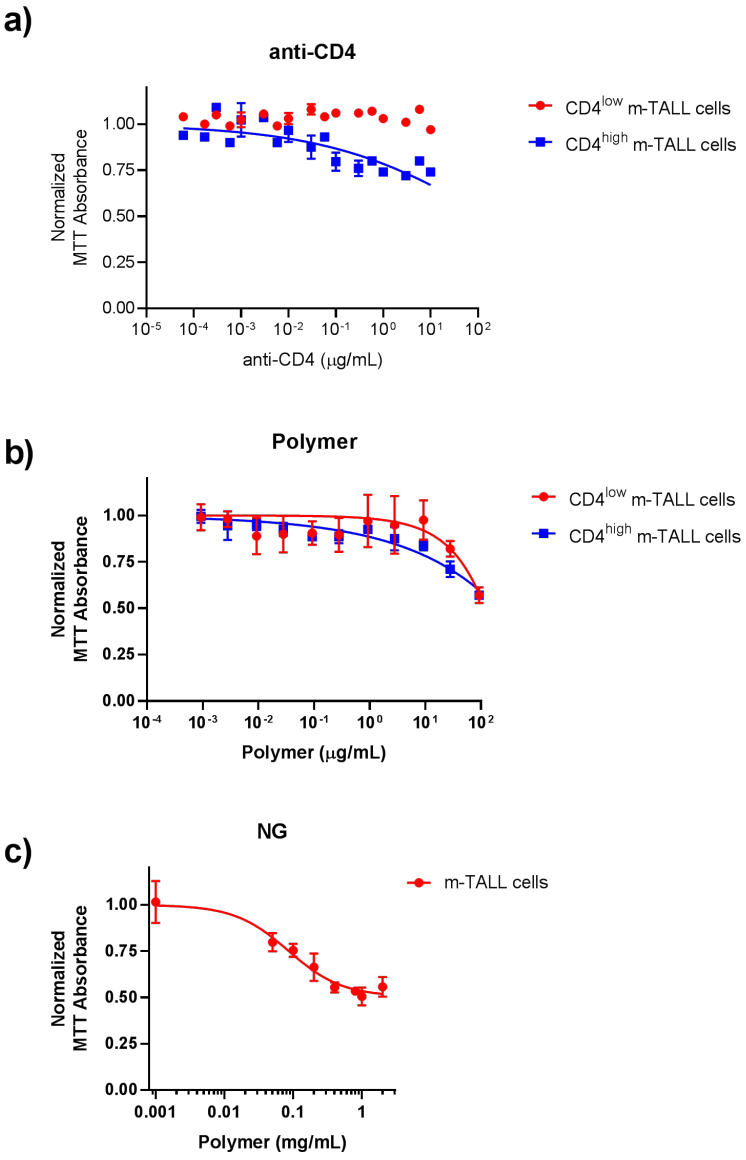


Figure S13: MTT assay results of mT-ALL cells after treatment with anti-CD4 (a), polymer (b) and NG (c).

Table S1: Extinction coefficients of IgG and NHS-PEG-PDS linker.

	λ_{280nm}	λ_{343nm}
IgG	199,500 $M^{-1}cm^{-1}$	-
NHS-PEG-SH	21,129 $M^{-1}cm^{-1}$	-
pyridine-2-thiol	-	8080 $M^{-1}cm^{-1}$

Table S2: Calculated DM1 IC_{50} values

	IC_{50} for DM1 (nM)	
mT-ALL cell	**$CD4^{low}$**	**$CD4^{high}$**
Polymer-DM1	223 ± 39	210 ± 34
NG-DM1	21 ± 2	26 ± 2
Anti-$CD4^{PEG}$/NG-DM1	1083 ± 71	233 ± 26
Anti-CD4-DM1	255 ± 42	37 ± 16

References

(1) Ghosh, S.; Basu, S.; Thayumanavan, S. Simultaneous and Reversible Functionalization of Copolymers for Biological Applications. *Macromolecules* **2006**, *39* (17), 5595–5597.

(2) Ryu, J. H.; Bickerton, S.; Zhuang, J.; Thayumanavan, S. Ligand-Decorated Nanogels: Fast One-Pot Synthesis and Cellular Targeting. *Biomacromolecules* **2012**, *13* (5), 1515–1522.