SUPPLEMENTARY MATERIAL

A NOVEL BIOCOMPATIBLE POLYMER DERIVED FROM D-MANNITOL USED AS A VECTOR IN THE FIELD OF GENETIC ENGINEERING OF EUKARYOTIC CELLS

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S.1. Experimental Section

S1.1. Synthesis and Characterization Data

Commercial reagents used in the synthesis of the polymer PUMan (D-mannitol, dithiotiethanol, sodium hydride, allyl bromide, p-toluenesulfonic acid, 2-aminoethane-1-thiol, and AIBN), all of them of analytical grade (P.A.), were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain) and used without further purification. Solvents of high purity grade were dried by appropriate standard procedures when necessary.

The new synthesized compounds were characterized by the usual techniques. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer equipped with ATR. 1H and 13C NMR spectra were recorded using a Bruker AV-300 or a Bruker Advance AMX-500 at the Centro de Investigación, Tecnología e Innovación of the University of Seville (CITIUS). Chemical shifts (δ) are reported as parts per million downfield from tetramethylsilane (Me4Si). Two-dimensional shift correlation spectra, such as 1H – 1H homonuclear and 13C – 1H heteronuclear were also recorded with the COSY and HETCOR pulse sequences, respectively. Elemental analyses were carried out in the Microanalysis Laboratories of the CITIUS Service at the University of Seville. The mass spectra were performed at the CITIUS mass spectrometer service in a Q-Exactive and Elite mass spectrometer from Thermo Fisher Scientific, with electrospray ionization (ESI-MS). For molecular weights determined by gel permeation chromatography (SEC/GPC) a Waters equipment provided with a refractive-index detector 2414 (thermostated at 40 ºC) was used. *N,N*-dimethylformamide containing LiBr (5.8 mM solution) or Na_2SO_4 (100 mM) pH 4 were used as the mobile phases. Samples (100 μL of 0.1% (w/v) solution) were injected and chromatographed with a flow of 1 mL min^{-1}. Waters Styragel or Waters Hydrogel columns were used, thermostated at 60 ºC. Molar mass averages and their distributions were estimated against polystyrene or polyethylene oxide standards. The thermal behavior of the polyurethanes was examined by differential scanning calorimetry (DSC) using a TA DSC Q200 Instrument calibrated with indium. Samples of about 2–3 mg were heated at a rate of 10 °C min^{-1} under a nitrogen flow of 50 mL min^{-1}, and cooled to −35 °C. The melting temperature (T_m) was taken as the maximum of the endothermic peak appearing on heating traces recorded at 10ºC min^{-1}, and the glass transition temperature (T_g) was taken as the temperature for the inflection point seen on heating traces recorded at 20 ºC min^{-1} from samples quenched from the melt. Thermogravimetric analyses (TGA) were performed with a TA SDT Q600 thermo balance. Polymer samples with a weight of around 3–4 mg were heated at a rate of 20 ºC min^{-1} within the temperature range of 30–600 ºC under an inert atmosphere. The polymerization reaction assays were performed in the absence of humidity, under an inert

atmosphere. All glassware was heated overnight at 80 ºC before use. The pure monomers were dried under vacuum and stored under an inert atmosphere until required.

The cationic polyurethane PUMan was obtained from the tetra-*O*-allyl derivative of D-mannitol as follows (see Schemes S1 and S2).

Synthesis of Monomers

2,3,4,5-Tetra-O-*allyl-1,6-di*-O-*trityl*-D-*Mannitol* **(TrMAL)**. To sodium hydride (60% p/p, 0.86 g, 21.6 mmol) in dry tetrahydrofuran (THF) (5 mL), previously washed with dry pentane (21 mL x 3) under argon atmosphere, a solution of 1,6-di-*O*-trityl-D-mannitol (TrM) [1] (3.28 g, 4.92 mmol) in dry THF (21 mL) was added dropwise at 0 °C. The mixture was stirred at 30 °C for 1 h before de addition of allyl bromide (1.87 mL, 21.65 mmol) and the stirring was maintained for 24 h at 30 °C. Then methanol (2.6 mL) was added dropwise, and the mixture was stirred for 1 h. The reaction mixture was evaporated to dryness and the residue was dissolved in CH_2Cl_2-H_2O 1:1 (100 mL). The organic phase was washed with water (30 mL), dried with $MgSO_4$ and concentrated to dryness. The residue was purified by flash column chromatography (1:9 *tert*-butylmethyl ether-hexane) to give the title compound as a white solid (3.1 g, 77%). M. p. 71-73 °C. IR: ν_{max} (cm^{-1}) 2962 (C-H vinyl), 1736 (C=C vinyl), 1541, 1489, 1457 (C=C arom), 1259 (C-O-C). ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) 3.08-3.14 (dd, 2H, H-$1_{a,}$ H-$6_{a,}$, *J*= 4.01 y 10.4 Hz), 3.54-3.59 (dd, 2H, H-$1_{b,}$ H-6_b, *J*= 1.9 y 10.3 Hz), 3.60-3.65 (m, 2H, H-2, H-5), 3.84-3.94 (m, 6H, 3 C*H*$_2$-CH=CH_2), 4.08-4.14 (d, 2H, H-3, H-4, *J*= 8,0 Hz), 4.24-4.31 (dd, 2H, C*H*$_2$-CH=CH_2, *J*= 12.6 y 5.3 Hz), 4.85-4.96 (m, 4H, 2 CH=C*H*$_2$), 5.20-5.46 (m, 4H, 2 CH=C*H*$_2$), 5.50-5.61 (m, 2H, 2 C*H*=CH_2), 5.97-6.08 (m, 2H, 2 C*H*=CH_2), 7.25-7.37 (m, 30H, Ph). ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) 62.3 (C-1, C-6), 71.0, 73.4 (4 *C*H_2-CH=CH_2), 77.8 (C-3, C-4), 78.6 (C-2, C-5), 86.6 (*C*Ph_3), 116.1 (CH=*C*H_2), 127.1, 127.9, 129.1 (Ph), 135.2, 135.5 (*C*H=CH_2), 144.4 (Ph quaternary C). HRMS: Calculated molecular weight for $C_{56}H_{58}O_6$ $(M + Na)^+$: 849.4098; experimental molecular weight: 849.4126. Anal. Calcd. for $C_{56}H_{58}O_6$: C, 81.32; H, 7.07. Found: C, 81.61; H, 7.11.

2,3,4,5-Tetra-O-*allyl*-D-*Mannitol* **(MAL)**. To a solution of TrMAL (3.43 g, 4.1 mmol) in 1:1 dichloromethane-methanol (100 mL), a solution of *p*-toluenesulfonic acid (2.14 g, 12.4 mmol) in 1:1 dichloromethane-methanol (70 mL) was added dropwise. The reaction mixture was stirred for 24 h at room temperature and then $NaHCO_3$ (3.75 g, 44.6 mmol) was added, and the stirring was continued for 1 h. After concentrating the mixture to small volume, the formed solid was filtered through siliceous earth and the filtrated was concentrated to dryness. The obtained residue was purified by flash column chromatography (1:2 Ethyl acetate-hexane) to

give MAL as a colorless oil (1.1 g, 79%). IR: ν_{max} (cm^{-1}) 3649 (O-H), 2962 (C-H vinyl), 1735 (C=C vinyl), 1259 (C-O-C), 1016 (C-OH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) 2.21-2.30 (m, 2H, OH), 3.52-3.61 (m, 2H, H-2, H-5), 3.68-3.78 (m, 4H, H-1_a, H-3, H-4, H-6_a), 3.84-3.93 (m, 2H, H-1_b, H-6_b), 3.94-4.27 (m, 8H, 4 CH_2-CH=CH_2), 5.08-5.34 (m, 8H, CH=CH_2), 5.84-5.96 (m, 4H, CH=CH_2). ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) 60.3 (C-1, C-6), 70.5, 73.9 (4 $C$$H_2$CH=$CH_2$), 78.5 (C-3, C-4), 79.5 (C-2, C-5), 117.1 (4 CH=CH_2), 134.7, 134.9 (4 CH=CH_2). HRMS: Calculated molecular weight for $C_{18}H_{30}O_6$ $(M + Na)^+$: 365.1898; experimental molecular weight: 365.1930. Anal. Calcd. for $C_{18}H_{32}O_7$: C, 59.98; H, 8.95. Found: C, 62.00; H, 8.78.

Tert-*butyl (2-mercaptoethyl)carbamate* **(BocCis).** This compound was obtained as previously described [2].

Synthesis of Polyurethanes

(MAL)DTDI. A round-bottom flask was loaded with MAL (1.11 g, 3.25 mmol) and the system was treated with three cycles of vacuum-argon before the addition of dry THF (2.2 mL). The mixture was stirred to get a solution and then dithiodiethyldiisocyanate (DTDI) [3,4] (0.66 g, 3.25 mmol), added under inert atmosphere, followed by one drop of the catalyst [dibutyltin (II) dilaurate]. The stirring was maintained during 24 h at 40 °C. After this time, the reaction mixture was treated with *tert*-butyl alcohol (0.97 mL) for 30 minutes and added dropwise into cold diethyl ether (200 mL). Finally, the precipitated polymer was filtered, washed with diethyl ether, and dried under vacuum at 40 °C for 24 h, to afford the title compound (1.6 g, 88%). IR: ν_{max} (cm^{-1}) 3337 (NH), 1698 (C=O), 1523 (N-H), 1251 (N-CO-O). ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm) 2.72-2.91 (m, 4H, CH_2S, terminal OH), 3.22-3.35 (m, 4H, CH_2NH), 3.39-4.60 (several m, 16H, H-$1_{a/b}$, H-2, H-3, H-4, H-5, H-$6_{a/b}$, OCH_2-CH=CH_2), 5.04-5.33 (m, 8H, CH=CH_2), 5.81-5.99 (m, 4H, CH=CH_2), 7.37 (s, 2H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm) 37.7 (CH_2S), 39.8 (CH_2NH), 58.7, 62.2 (C-1, C-6), 69.8, 73.0 (O$C$$H_2$-CH=$CH_2$), 76.9, 77.5, 77.8, 79.1 (C-2, C-3, C-4, C-5), 115.9, 116.1 (CH=CH_2), 135.3, 135.7 (CH=CH_2), 156.3 (CO). Mn molar mass 3840 g/mol (calculated by ^{1}H NMR).

(MBocCis)DTDI. The compound (MAL)DTDI (200 mg, 0.37 mmol) was solved in DMF (1.2 mL), and the obtained solution was deoxygenated under a stream of argon for 30 minutes. Then, BocCis (1.04 g, 7.85 mmol) and AIBN as initiator (0.20 g, 1.18 mmol) were added under inert atmosphere and the mixture was stirred at 80 °C for 24 h. After this time, the obtained solution was added dropwise in cold diethyl ether (300 mL). The precipitated polymer was filtered and subsequently purified by dissolving in dichloromethane and reprecipitation in diethyl ether. The solid was filtered, washed with diethyl ether and dried under vacuum at 40 °C

for 24 h to afford (MBocCis)DTDI (160 mg, 96%). IR: ν_{max} (cm^{-1}) 3339 (NH), 1669 (C=O), 1509 (N-H), 1269 (N-CO-O). ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm) 1.38 [bs, 36H, C(CH_3)$_3$], 1.69-1.81 (m, 8H, OCH$_2$CH$_2$CH_2S), 2.43-2.57 (m, 16H, SCH_2CH$_2$NHBoc, OCH$_2$CH_2CH$_2$S), 2.63-2.65 (m, terminal OH), 2.71-2.85 (m, 4H, CH$_2$S-S), 3.01-3.14 (m, 8H, SCH$_2$CH_2NHBoc), 3.16-3.23 (m, 4H, S-S-CH$_2$CH_2NH), 3.34-3.82 (m, 12H, H-2/H-3/H-4/H-5, OCH_2CH$_2$CH$_2$S), 3.87-4.52 (m, 4H, H-1/H-6), 6.82, 6.92, (bs, 4H, NHBoc), 7.95 (bs, 2H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm) 28.2 (CH$_3$), 30.7 (OCH$_2$*C*H$_2$CH$_2$S), 35.7 (CH$_2$S, SCH$_2$), 37.8 (CH$_2$S-S), 38.7 (CH$_2$NH), 41.1 (CH$_2$NHBoc), 64.7 (C-1/C-6), 70.1 (OCH$_2$), 76.0, 76.7 (C-2/C-3/C-4/C-5), 77.7 [*C*(CH$_3$)$_3$], 154.6 (C=O Boc), 162.2 (C=O chain). Mn molar mass 8100 g/mol (calculated by ^{1}H NMR).

PUMan. Polyurethane (MBocCis)DTDI (0.24 g, 0.19 mmol) was treated with a 4M HCl solution in dry dioxane (18.6 mL) and the resulting mixture was stirred at room temperature for 4 h, under inert atmosphere. Then the reaction mixture was filtered, and the product obtained was dried under vacuum at 40 °C for 24 h to afford PUMan (106 mg, 66 %). IR: ν_{max} (cm^{-1}) 3359 (N-H), 2898 (ammonium), 1696 (C=O), 1500 (N-H), 1251 (N-CO-O). ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm) 1.67-1.86 (m, 8H, OCH$_2$CH_2CH$_2$S), 2.54-2.86 (m, 16H, CH$_2$S, terminal OH), 2.91-3.20 (m, 16H, CH$_2$S-S, CH_2NH$_2$, CH_2NH), 3.32-3.72 (m, 12H, OCH_2CH$_2$CH$_2$S, H-2, H-3, H-4, H-5), 3.88-4.03 (m, 2H, H-1$_a$, H-6$_a$), 4.35-4.58 (m, 2H, H-1$_b$, H-6$_b$), 7.43 (bs, 2H, NH), 8.33 (bs, 8H, NH_3^+). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm) 27.4, 27.7 (OCH$_2$CH$_2$*C*H$_2$S), 29.6 (S*C*H$_2$CH$_2$$NH_3^+$), 33.8 (OCH$_2$*C*H$_2CH_2$S), 37.8 (CH$_2$$NH_3^+$), 38.0, 38.3 (CH$_2$S-S), 41.7 (C$H_2$NH), 63.4 (C-1, C-6), 66.3 (OCH_2CH$_2$CH$_2$S), 77.1 (C-2, C-3, C-4, C-5), 154.8 (CO). Mn molar mass 5500 g/mol (calculated by ^{1}H NMR).

S1.2. Fluorescence Measurements

Emission spectra were performed to quantify the interaction between the PUMan polymer and DNA (ctDNA and plasmids). Measurements were performed in a Hitachi F-2500 spectrofluorimeter interfaced to a PC for the recording and handling of the spectra. A standard fluorescence quartz cell of 10 mm path length was used. Emission intensities of the PUMan/ DNA polyplex solutions were measured in the presence of ethidium bromide (EB) at different polymer concentrations maintaining constant the concentrations of both EB and DNA ([EB]= $2.4x10^{-5}$ mol dm^{-3} and [DNA]=$3x10^{-5}$ mol dm^{-3} for ctDNA and plasmids). The polymer concentration was varied depending on the N/P ratio desired. The excitation and emission wavelengths used were 480 nm and 590 nm, respectively. The lamp voltage was 400 V and the excitation and emission slits were set at 5 nm. The measurements were repeated at least 5 times.

S1.3. Zeta Potential Measurements

Zeta-potential (ζ) values were obtained measuring the electrophoretic mobility of the sample from the velocity of the particles using a Laser Doppler velocimeter (LDV). The experiments were carried out with a Zetasizer Nano ZS Malvern Instrument Ltd (Malver, Worcestershire, UK). A DTS1060 polycarbonate capillary cell was used. The measurements were repeated 5 times.

S1.4. Dynamic Light Scattering Measurements (DLS)

The size and the polydispersity index of the different polyplexes prepared were obtained from DLS measurements by using a Zetasizer Nano ZS Malvern Instrument Ltd (UK). Samples were illuminated with a laser, at a fixed detection arrangement of 90º to the center of the cell area, and fluctuations in the intensities of the scattered light were analyzed. The obtained results were the average of 10 measurements.

S1.5. Circular Dichroism Spectra

Electronic circular dichroism (CD) spectra were recorded in a Biologic Mos-450 spectropolarimeter. A standard quartz cell of 10 mm path length was used. Spectra of DNA in the presence and absence of polymer was performed at different N/P ratio. Each spectrum was obtained from an average of 10 runs with an equilibrium time of 5 min before each scan. The spectra obtained were expressed in terms of ellipticity, Θ_{obs}.

S1.6. Agarose Gel Electrophoresis

Agarose gel electrophoresis of the Phyco69 and pEGFP-C1 plasmids and of the PUMan/DNA polyplexes were performed at different N/P ratios.

The gel was prepared at 1% (p/v) in a buffer Tris-Acetate-EDTA (TAE), obtained from a stock TAE 50X solution. The intercalative fluorophore ethydium bromide 0.1% was used as staining agent for the visualization of the double-stranded DNA. Electrophoresis measurements were carried out at 60 V for a period of time of 60 min. Imaging treatment was performed by a transilluminator Vilver Loumat FLX20 equipped with 6 x15W -312nm tubes. All lanes had 600 ng of the plasmid ([Plasmid]=60 ng μl^{-1}). The mass of polymer added depended on the N/P value desired for each polyplex.

The pEGFP-C1 and Phyco69 plasmids were linearized by digestion with *EcoRI* to determine the number of base pairs contained in them. Plasmids were propagated in E. coli (DH5α) and purified using silica-based membrane in columns (GeneJET plasmid kit) acquired from Thermo Scientific following the indications of the manufacture and eluted in Tris buffer 20 mM.

S1.7. Atomic Force Microscopy (AFM)

Atomic force microscopy is a technique used to obtain information about the structure of PUMan/Phyco69 and PUMan/pEGFP-C1 polyplexes. AFM images were obtained with a Molecular Imaging PicoPlus 2500 AFM (Agilent Technologies). Silicon cantilevers (Model Pointprobe, Nanoworld) with a resonance frequency of around 240 KHz and nominal force constant 42 N/m were used. All images were recorded in the tapping mode, with scan speeds of about 0.5 Hz and data collection at 256x256 pixels.

Plasmid DNA dilute solutions (1.5 μmol dm^{-3}) were used due to the large size of the polynucleotide. 30 μL of polyplex (or free plasmid DNA) solution were deposited onto modified mica, incubated for 30 min, washed with pure water and air dried for AFM imaging. The modified mica surface was prepared dropping a 0.1% (v/v) APTES aqueous solution onto a freshly cleaved mica surface. After 20 minutes, the surface was washed with ultra-pure water and air dried [5].

S1.8. In Vitro Assays

Cytotoxicity and phytotoxicity measurements of PUMan were carried out with different mammalian cells and the microalga *C. reinhardtii*, respectively, at different concentrations of polymer.

The cytotoxic activity of the polymer was studied by using the MTT assay [6]. Cell lines were plated out into 96 well plates at a density of 3000 cells per plate. Five human cancer cell lines and a normal cell line were used: A549 (adenocarcinomic human alveolar basal epithelial cell line), HepG2 (human liver cancer cell line), LS180 (adenocarcinomic human colonic epithelial cell line), MCF7 (breast cancer cell line) and RPE-1 (normal cell line). Different doses of polymer were added to the wells and the plate returned to the incubator for four more days. Later, they were pulsed with MTS (ROCHE). Cell viability was measured by luminometry. Each dose point was measured in triplicate.

Phytotoxicity assays of PUMan in the microalga *Chlamydomonas reinhardtii* were performed at different polymer concentrations. *C. reinhardtii* cultures grown in TAP liquid medium were harvested at the exponential phase of growth and 100-fold concentrated by centrifugation for 5 min at 10000g. 10 μL drops of the concentrated cell suspension were spotted on multi-well plates with agar solidified-TAP medium and growing concentrations of PUMan and incubated at 25ºC and 100 uE m^{-2} s^{-1} in a growth chamber. The cellular viability was followed 24 h after inoculation.

S1.9. Transfection Assays

PUMan was used as vehicle to transfect plasmid DNA into human tissue cultured cells. The pEGFP-C1 plasmid was used for these experiments. This plasmid carries a GFP coding sequence with the necessary regulatory elements for constitutive expression of the gene in human cells. Transfection assays were carried out with the U2OS cell line (from human osteosarcoma). This is an easy-to-transfect cell line frequently used in human molecular and cellular biology studies. Besides, U2OS has neither adenoviral infections nor large T antigen so that it is chosen for this type of experiments. U2OS cells were grown in DMEM medium with 10% fetal bovine serum at 37 ºC in 5% CO_2 atmosphere.

In our experimental set up 3 μg of plasmid DNA was added to a solution containing 180 μL of Optimem and 24 μL of PUMan (1μg/μL) buffered solution (HEPES 10 mM, pH=7.4). The mixture was incubated at room temperature for 5 min and, subsequently, added to a 50% confluent 6 cm plate with 3 mL of DMEM medium. As negative control, the cells were transfected with a mixture of plasmid DNA and Optimem. As positive control FuGENE 6 transfection reagent was used according to the manufacturer's protocol (i.e. 3 μg of plasmid DNA in 200 μL Optimem plus 9 μL of FuGENE 6). Transfection efficiency was evaluated by fluorescence microscopy.

A determined quantity of the phospholipid 1,2-dioleoyl-sn-glycer-3-phosphoethanolamine (DOPE) was added to the polyplex mixture to improve the transfection efficiency of PUMan. The procedure used for transfection assays was the same than that used in the absence of DOPE.

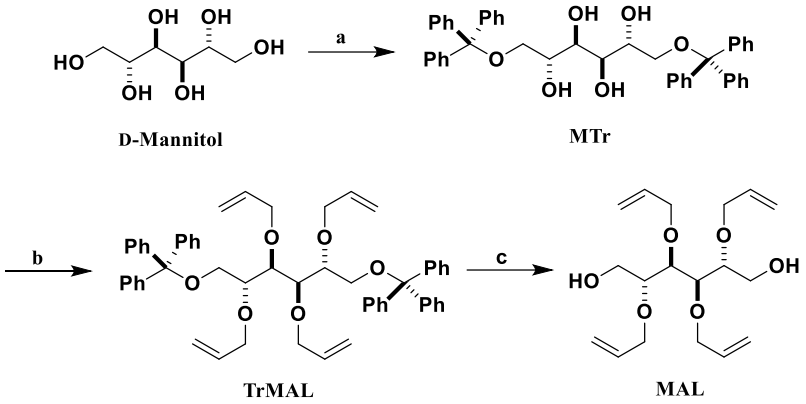
S1.10. Chlamydomonas reinhardtii Nuclear Transformation

Nuclear transformation of *C. reinhardtii* was carried out using the glass beads method of Kindle [7] with minor modifications. *Chlamydomonas* cultures were grown to a cell density of about 10^7 cells per mL, spinned down and resuspended to get a final 100-fold concentrated cell suspension. 0.3 g of sterile glass beads (0.4-0.6 mm Ø) were added to 0.6 mL of concentrated cell suspension and to 0.2 mL of the polyplex PUMan/Phyco69 with the desired quantities of polymer (8.1 μM for N/P=10 and 13.7 μM for N/P=17) and plasmid (2.3μM). A constant mass of 0.2 μg of plasmid per transformation reaction was used in all the assays. Plasmid Phyco69 has the *APHVIII* gene from *Streptomyces rimosus* encoding an aminoglycoside 3'-phosphotransferase enzyme which confers resistance to the antibiotic paromomycin [8]. This mixture was agitated during 10 s and then cells were resuspended in 50 mL of fresh TAP medium and left in dim light overnight. After this incubation, cells were spread onto the selective solid medium carrying 30 μg mL^{-1} of paromomicyn. Transformed colonies were visible after 4 or 5 days.

S.2. Results and Discussion

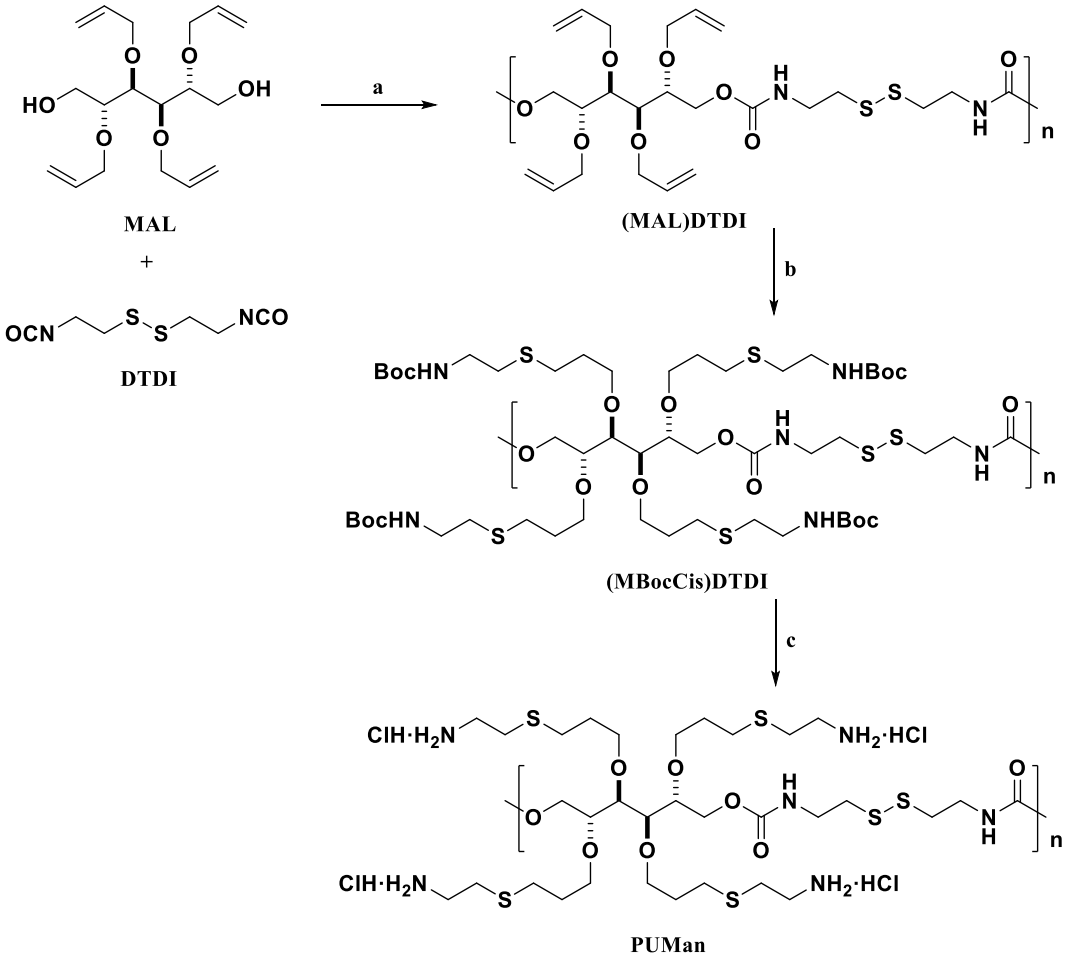
S2.1. Characterization of Monomers and Polyurethanes

The monomer 2,3,4,5-tetra-O-allyl-D-mannitol (MAL) was synthesized from the intermediate compounds MTr and TrMAL in good yield (Scheme S1).



Scheme S1. Synthesis of 2,3,4,5-tetra-*O*-allyl-D-mannitol (MAL). (a) Ph_3CCl, Pyridine, r.t.; (b) NaH, Tetrahydrofurane, Allyl bromide, 30 ºC; (c) *p*Ts-SO_3H, CH_2Cl_2-MeOH 1:1, r.t.

The new polyurethane PUMan was prepared (Scheme S2) by a polyaddition reaction of the 2,3,4,5-tetra-O-allyl-D-mannitol (MAL) and dithiodiethyldiisocyanate (DTDI) using dry tetrahydrofurane (THF) as solvent and dibutyltin (II) dilaurate as catalyst, under inert atmosphere at 40°C for 24 h. The multi-*O*-allyl PU was isolated in yield over 85%. Then, "click" thiol-ene reactions were carried out with *tert*-butyl(2-mercaptoethyl)carbamate (BocCis) in dry *N*,*N*-dimethylformamide (DMF), using 2,2'-azobisisobutyronitrile (AIBN) as initiator, at 80 °C under inert atmosphere, to afford (MBocCis)DTDI in high yields. The addition of BocCis to the allylic polymer was confirmed by ^{1}H NMR spectra due to the disappearance of the signals at δ 5.04-5.33 ppm and 5.79-5.99 ppm attributed to the *O*-allyl groups, as well as the appearance of new signals at δ 1.40 and δ 6.85-6.96 ppm corresponding to the CH_3 and NH groups, respectively, of the *N*-Boc protecting groups.



Scheme S2. Synthesis of PUMan by the poliaddition reaction of MAL and DTDI leading to (MAL)DTDI and subsequent derivatization by "click" thiol-ene reactions and *N*-deprotection. (a) Dibutyltin (II) dilaurate, THF, 40 °C. (b) *Tert*-butyl(2-mercaptoethyl)carbamate, DMF, AIBN, 80 °C. (c) 4M HCl in 1,4-dioxane, r.t.

Removal of the *N*-Boc protecting groups of (MBocCis)DTDI was carried out by their treatment with 4M HCl in dry dioxane, to afford the corresponding polycationic PU hydrochloride PUMan in good yield. The ^{13}C NMR spectra of cationic PU demonstrated the efficiency of the *N*-Boc deprotection by the disappearance of the signals at δ 28.3 ppm, δ 76.8 or 77.7 ppm and δ 155.3 ppm, attributed to the protecting group. Likewise, ^{1}H NMR spectra showed a new broad singlet at δ 8.34 ppm due to the NH_3^+ groups. IR spectra data showed an increase in the intensity of the absorption band at 2898 cm^{-1} due to N-H, confirming the presence of the ammonia groups of PUMan (Fig. S1).

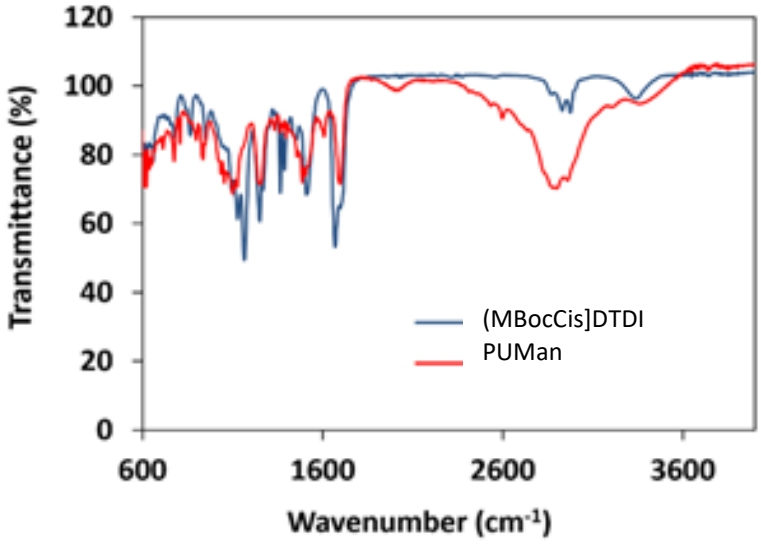
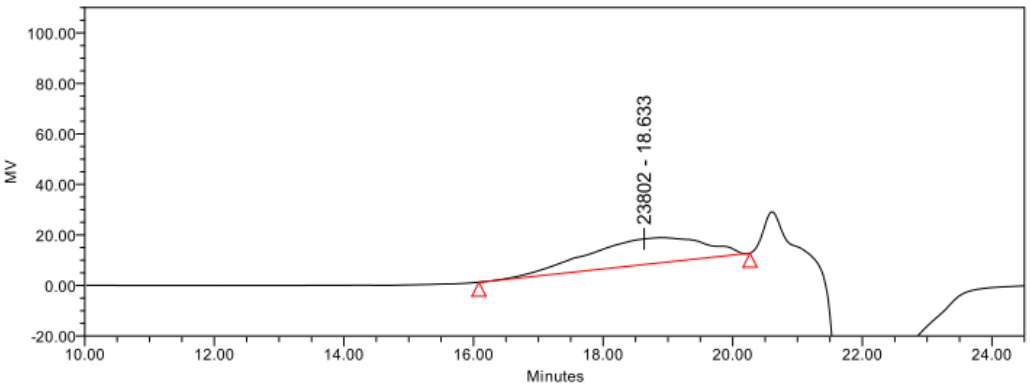


Fig. S1. IR spectra of PUMan and its precursor (MbocCis)DTDI.

The precursors (MAL)DTDI, (MBocCis)DTDI, and the cationic PUMan were analyzed by SEC/GPC to calculate the molar masses (Fig. S2-S4). Since these results were not reliable, ^{1}H NMR was used to calculate the Mn molar mass values. The terminal hydroxyl groups of the polyurethane chains were identified by treatment with D_2O, and then they were comparatively integrated in relation to the rest of the signals of the repetitive constitutional unit (RCU). By means of these calculations, a logical sequence of seven RCU was found, and it was done as follows. For PUMan the signal at δ 2.70-2.75 ppm assigned to the terminal OH groups was integrated in relation to the signals at δ 2.90-3.21 ppm corresponding to CH_2S-S, CH_2NH_2, and CH_2NH groups (Fig. S7). In the case of the precursor (MAL)DTDI the signal at δ 2.86-2.91 ppm assigned to the OH terminal groups was integrated in relation to the allyl signals at δ 5.04-5.33 ($CH=CH_2$) and 5.81-5.99 ($CH=CH_2$) (Fig. S5). For (MBocCis)DTDI the signal at δ 2.63-

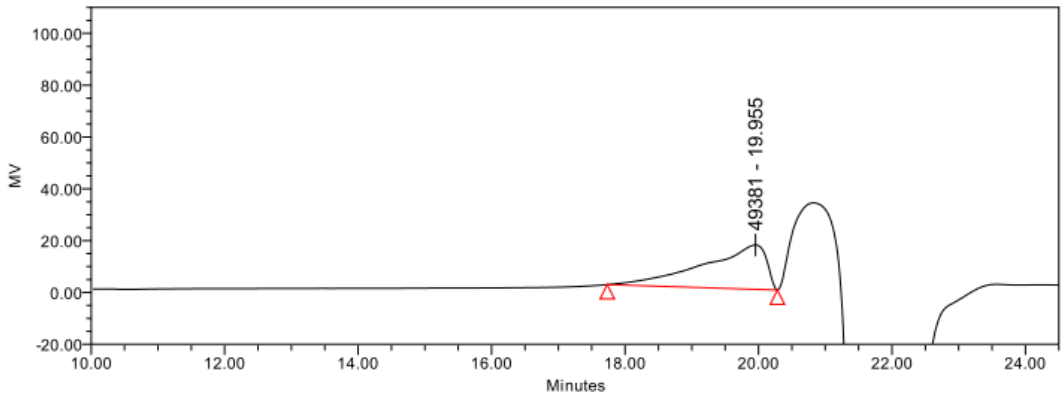
2.65 ppm assigned to the terminal OH groups was integrated in relation to the signals at δ 3.16-3.23 ppm corresponding to S-S-CH_2CH_2NH groups (Fig. S6). According to the data cited above the Mn calculated for the seven RCU for (MAL)DTDI, (MBocCis)DTDI, and PUMan were 3840, 8100, and 5500 g/mol, respectively.



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Polydispersity	MW Marker 1	MW Marker 2
1		26074	27103	23802	28565	30574		1.039487		

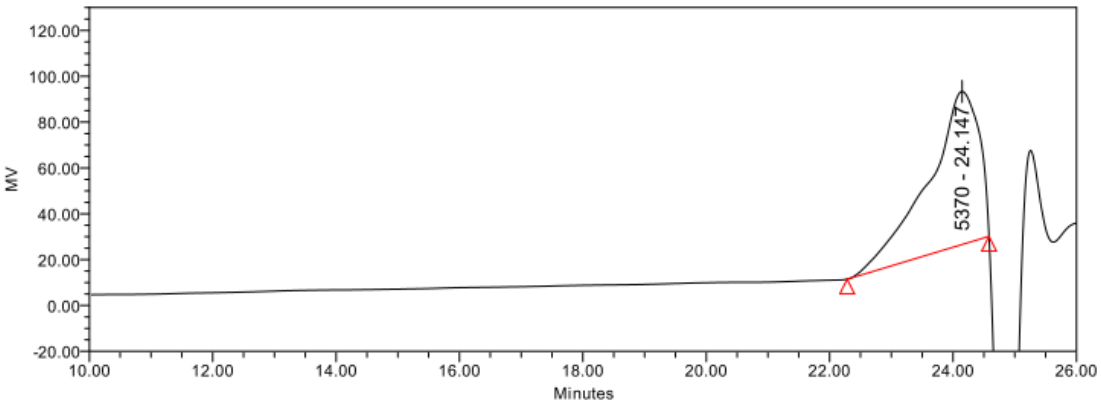
Fig. S2. SEC chromatogram of (MAL)DTDI using *N*,*N*-dimethylformamide containing LiBr 5.8 mM solution, against polystyrene standards.



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Polydispersity	MW Marker 1	MW Marker 2
1		34379	37487	49381	40777	43935		1.090385		

Fig. S3. SEC chromatogram of (MbocCis)DTDI using *N*,*N*-dimethylformamide containing LiBr 5.8 mM solution as the mobile phase, against polystyrene standards.



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Polydispersity	MW Marker 1	MW Marker 2
1		5971	6254	5370	6583	6953		1.047279		

Fig. S4. SEC chromatogram of PUMan using Na_2SO_4 (100 mM) pH 4 as the mobile phase against, polyethylene standards.

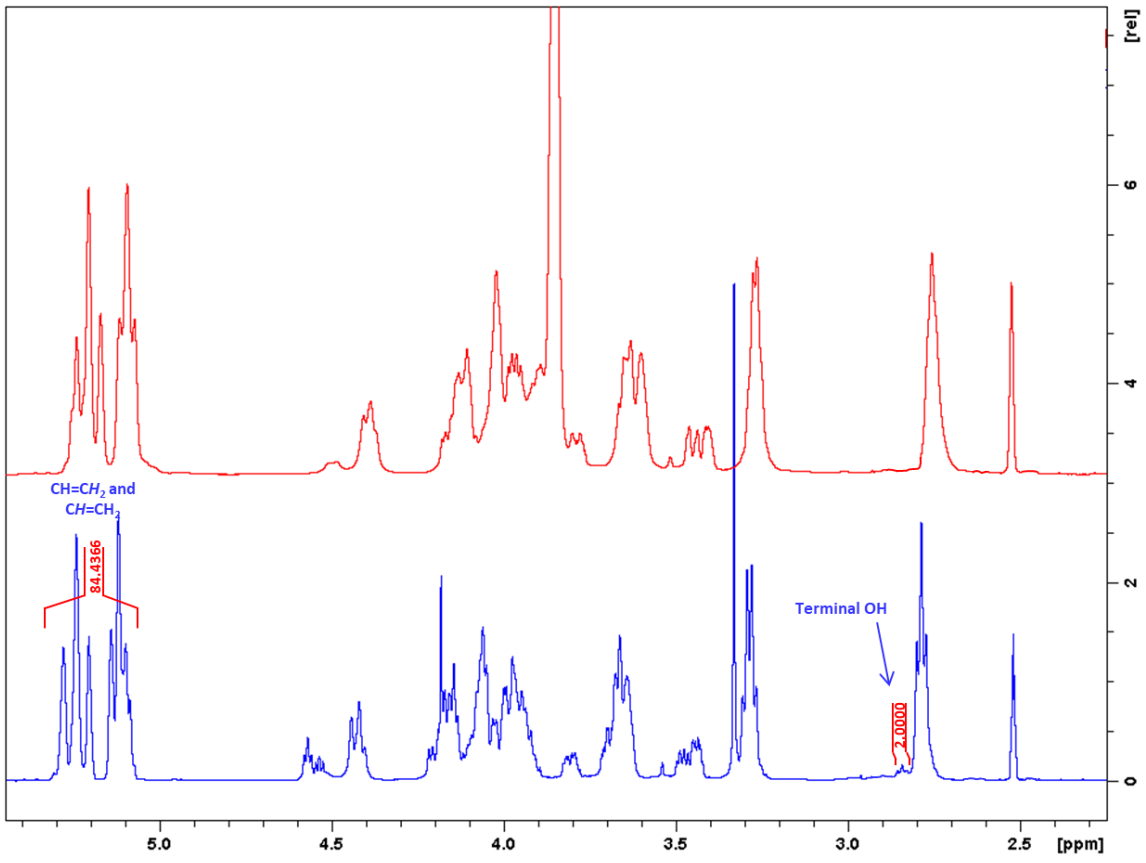


Fig. S5. ^{1}H NMR of (MAL)DTDI in DMSO-d_6 (blue) and DMSO-d_6/D_2O (red).

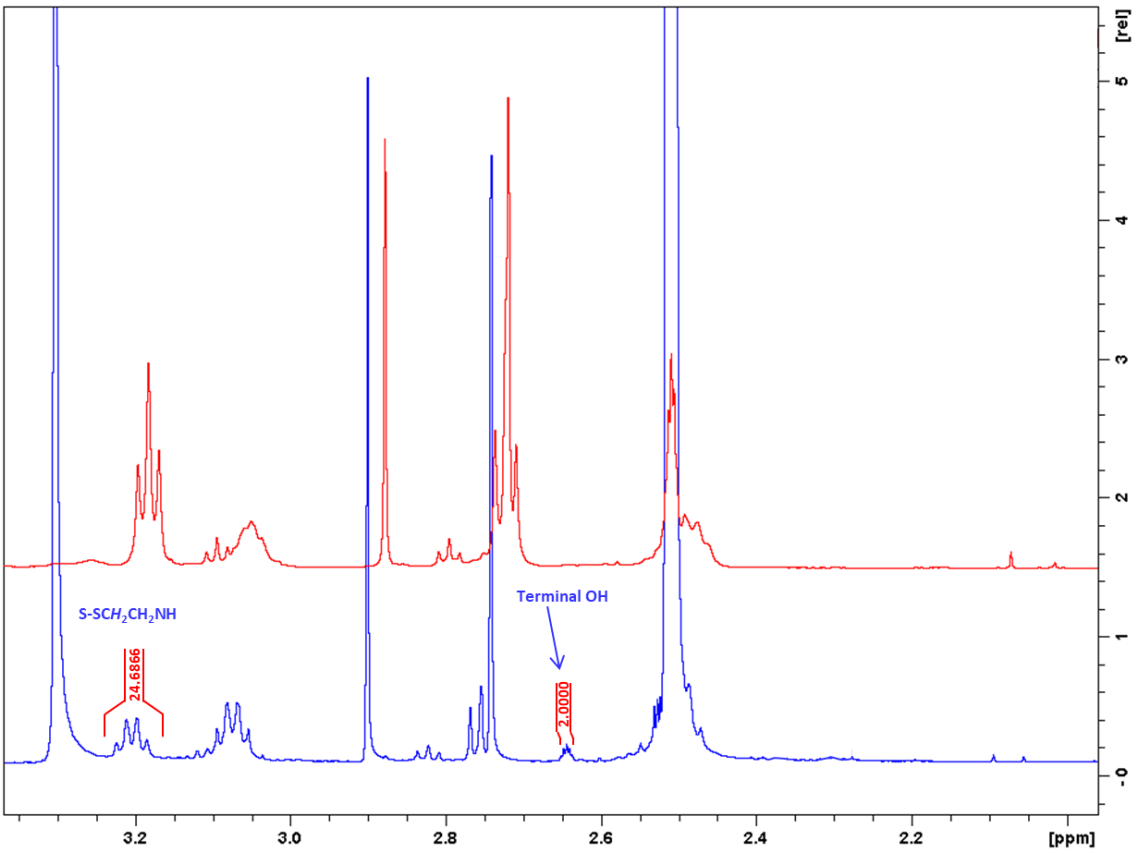


Fig. S6. ^{1}H NMR of (MBocCis)DTDI in DMSO-d_6 (blue) and DMSO-d_6/D_2O (red).

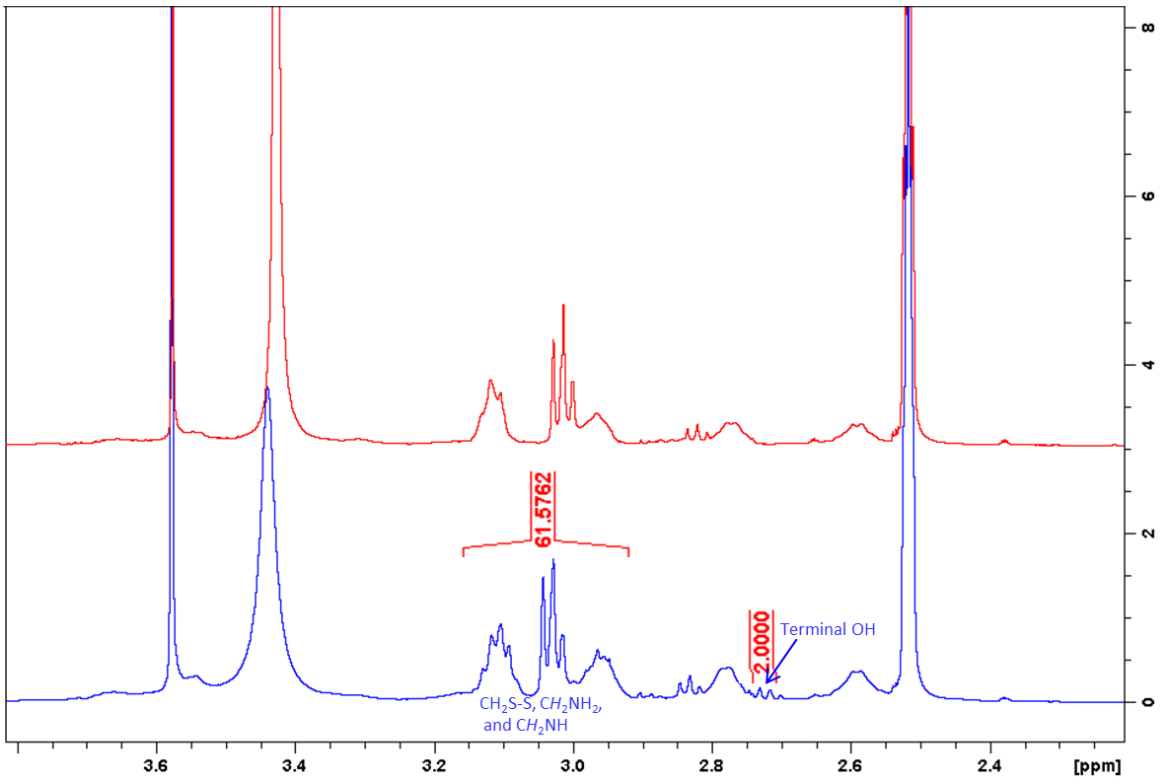


Fig. S7. ^{1}H NMR of PUMan in DMSO-d_6 (blue) and DMSO-d_6/D_2O (red).

The results of TGA and DSC thermal analysis of the PU described above are collected in Table S1. TGA studies of the *O*-allyl polymers showed thermal stability up to 188 °C for the D-mannitol derivative. The decomposition processes took place in two stages: the first one at temperatures between 256 and 259 °C, and the second one in the range 324-333 °C. The decomposition profile obtained for the cationic PUMan is shown in Fig. S8.

The DSC study of this PU showed T_g values ranging from 2 °C to 18 °C, corresponding the lowest values to cationic sample. Endothermic peak associated to melting processes were only observed for the *N*-Boc protected PU (MBocCis)DTDI around 115 °C. The rest of the PU manifested an amorphous behavior. They all presented low T_g values, in the range 2-18 °C, corresponding the lowest value 4 °C to the cationic PUMan.

Table S1. Thermal properties of PUMan and its precursors.

	TGA				DSC	
Polyurethane	T_d[a] (°C)	T_{ds}[b] (°C)	ΔW[c] (%)	T_g[d] (°C)	T_m[e] (°C)	ΔH_m[e] (J g^{-1})
(MAL)DTDI	188	257/328	34/51	14		
(MBocCis)DTDI	242	259/327	51/41	8	72/117	31/33
PUMan	242	258/332	40/52	2		

[a]Temperature at which 10% weight loss was observed in the TGA traces recorded at 10 °C min^{-1}. [b]Temperature for maximum degradation rate. [c]Remaining weight at 600 °C. [d]Glass transition temperature taken as the inflection point of the heating DSC traces of melt-quenched samples recorded at 20 °C min^{-1}. [e]Melting temperature (T_m) and respective enthalpy (ΔH_m) measured by DSC at a heating rate of 10 °C min^{-1}.

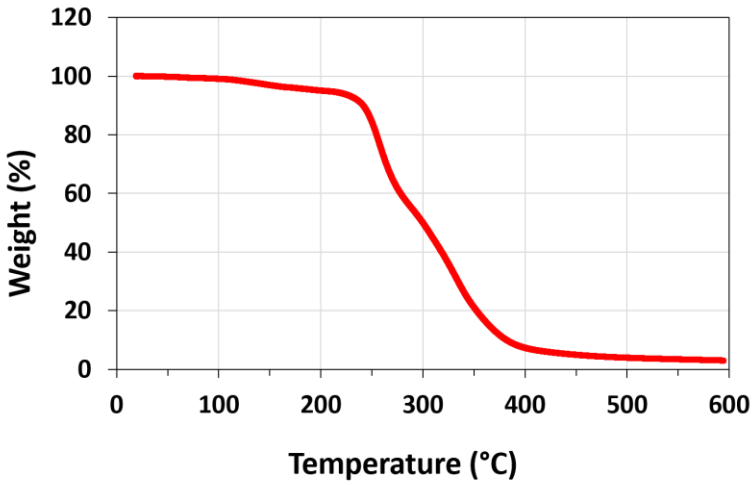


Fig. S8. Thermal degradation curve of PUMan under inert atmosphere. Weight (%) versus temperature.

2.2 Formation of the polyplexes PUMan/ctDNA

EB is an agent that spontaneously interacts with DNA by an intercalative mode. This is reflected in a strong emission intensity of the dye when their planar aromatic rings are located between the base pairs of the polynucleotide double helix [9,10]. Any change in the solution

environment, e.g., the addition of species interacting with nucleic acids, can cause the EB molecules to move from the intercalative position to a more polar environment, decreasing the emission intensity of the dye. This was observed when PUMan was added to an EB/ctDNA solution (Fig. S9A): a decrease in the EB emission intensity when the N/P ratio is increased, i.e. when the amount of polyurethane is increased. However, not all EB molecules are displaced into the solution since the emission intensity value obtained at the highest N/P ratio is still higher than that observed for the same EB concentration in the absence of DNA. Bearing in mind that the displacement of EB molecules in the presence of a cationic vector (PUMan here) is usually due to a conformational change in the secondary structure of the nucleic acid [11], the result obtained demonstrates that the PUMan polyurethane only provokes a partial conformational change in ctDNA.

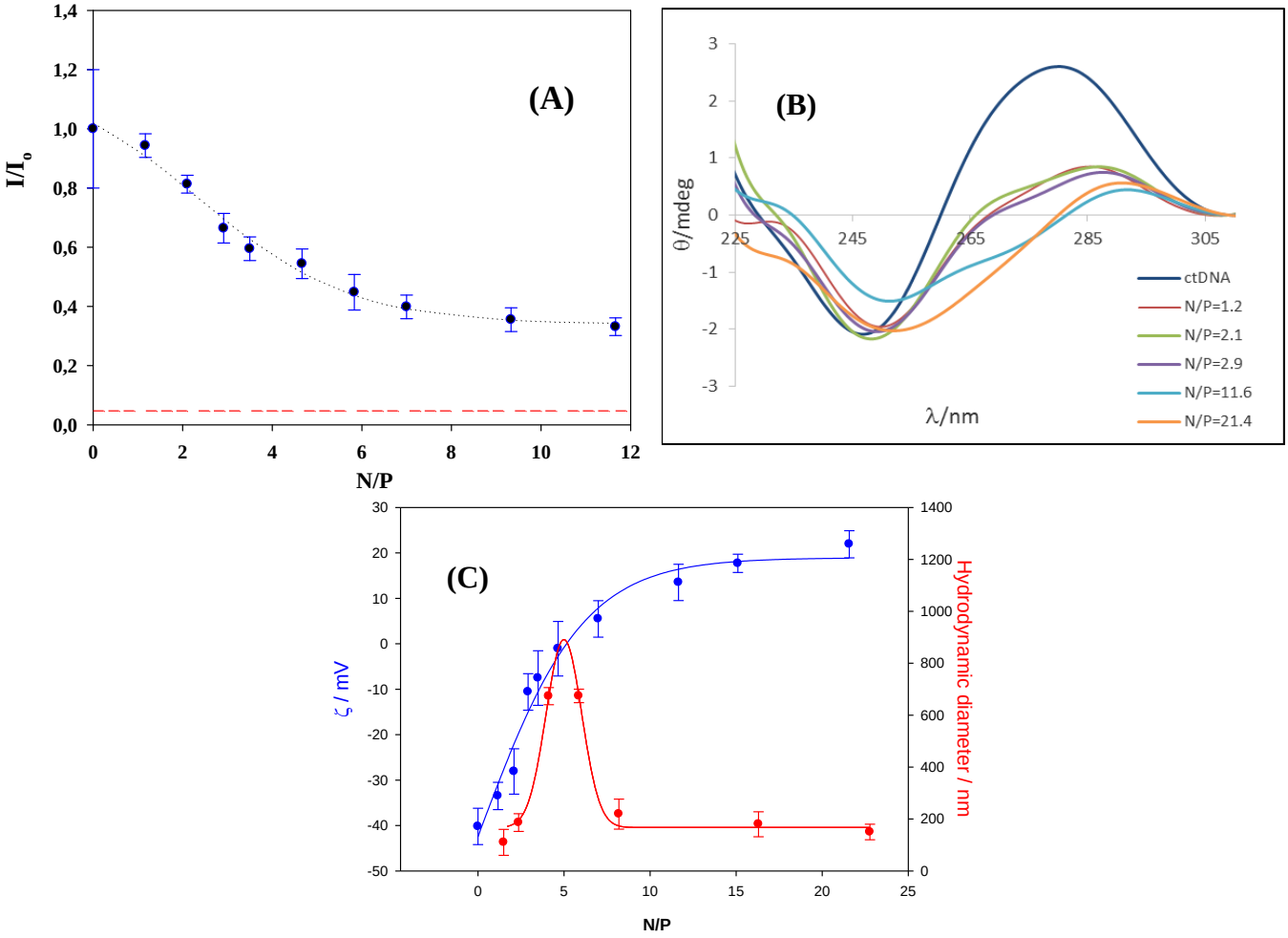


Fig. S9. (A) Plot of the relative emission intensity of EB versus the N/P ratio for ctDNA ([EB]=2.4x10^{-6} moldm^{-3}). Points correspond to experimental data at different N/P ratio and the red line shows the emission intensity of EB in the absence of ctDNA. (B) Circular dichroism spectra of ctDNA at different N/P ratio. (C) Zeta potential and hydrodynamic diameters of the polyplexes PUMan/ctDNA at different N/P ratios. Conditions for all the measurements: [DNA]=3x10^{-5} mol dm^{-3} HEPES 10 mmol dm^{-3}.

This partial displacement was confirmed by running circular dichroism spectra of ctDNA in the presence and absence of PUMan. The circular dichroism method provides information on the conformation of DNA and on the changes, it may undergo in the presence of different kinds of vectors [11-14]. Fig. S9B collects the ctDNA spectra obtained at different N/P values. A decrease of the typical bands of the polynucleotide (a negative band due to the helicity of the double-strand centered at 240 nm and a positive one at 278 nm due to π–π stacking interactions between the base pairs) is observed when the PUMan concentration increases in the solution; that is, the polymer provokes a disruption in the base stacking. However, the secondary structure is not totally lost in the working condition studied. In fact, the band corresponding to the π–π stacking interactions between base pairs suffers a greater modification than that corresponding to the helicity of the double strands. This indicates that a partial condensation of the polynucleotide takes place in the presence of the polyurethane with the formation of PUMan/ctDNA polyplexes.

The CD spectra also show a movement of the bands toward higher wavelengths when the N/P ratio increases. This movement of the bands is related to the melting of the polynucleotide strands in the presence of PUMan, which demonstrates the interaction of the polymer with the nucleic acid but not the complete condensation of the latter.

To deepen in the study about the structural characteristics of the polyplexes, zeta potential and dynamic light scattering measurements were carried out. Fig. S9C shows the results obtained. The zeta potential increases from negative to positive values when the PUMan concentration augments at constant ctDNA concentration. This increase occurs until a plateau of about +20 mV is reaching at the highest polymer concentrations. In general, a change in the polyplex charge must occur to favor a conformational change in the polynucleotide. The observed potential change from about -40 mV to only +20 mV seems to be in agreement with the CD results obtained here, that is, a partial DNA conformational change in which the helicity of the double strands is only slightly modified takes place (according to previous works [15], a total condensation would give a final potential of about +30-40 mV for the plasmid concentrations used here).

With respect to the hydrodynamic radii measured of the PUMan/ctDNA complexes, a first increase in the size of the polyplexes with the N/P ratio was observed. An increase in the N/P value, or the same in the polymer concentration at constant DNA concentration, provokes a decrease in the charge of the polyplexes. This results in a diminution of electrostatic repulsions between polyplexes which favors their agglomeration. In fact, a maximum value of the diameter is observed at the N/P ratio closed to 5, the same N/P ratio at which a value of the zeta potential

close to zero was obtained. A further increase in the N/P ratio leaded to a decrease in the hydrodynamic diameter. As Fig. S9C shows, the charge of the polyplexes increases for N/P ratios higher than 5. Therefore, the electrostatic repulsions between polyplexes augment and their tendency to agglomerate decreases, this leading to a diminution in the polyplexes size. A diameter of approx. 200 nm was observed at the highest N/P ratio.

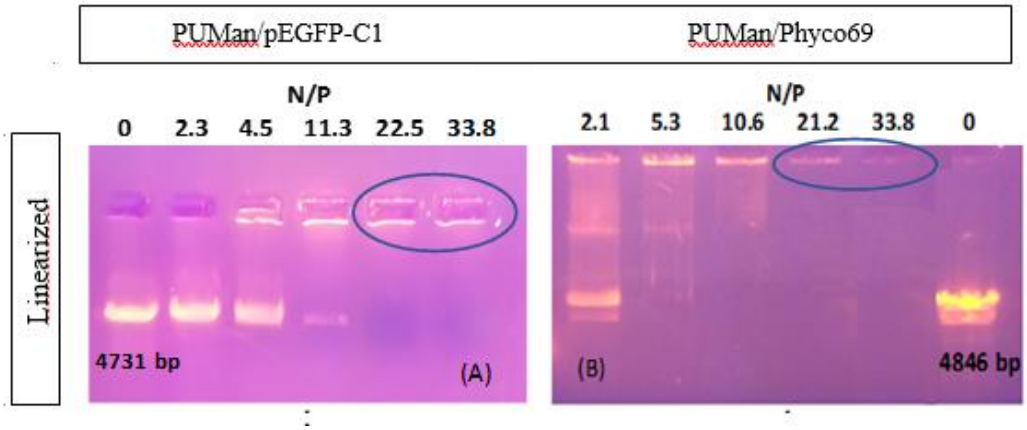


Fig. S10. Electrophoretic mobility of polyplexes PUMan/digested pEGFP-C1 and PUMan/digested Phyco69 at different N/P ratios. All wells were loaded with the same quantity of plasmid, [Plasmid]=60 ng/µl.

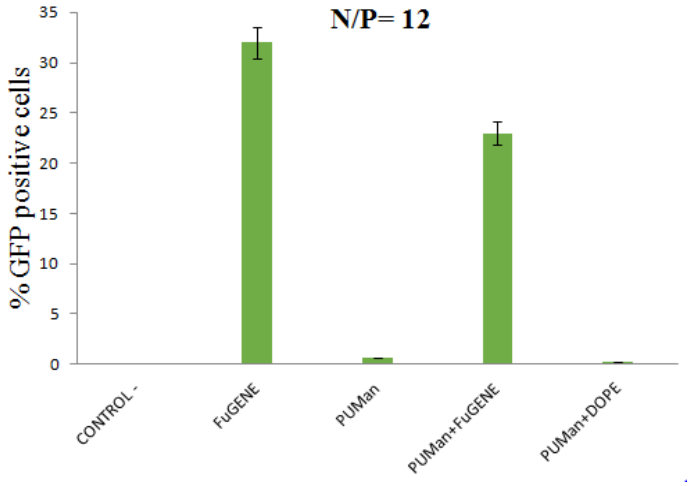


Fig. S11: Percentage of GFP positive cells after transfection with 3 µg of the plasmid pEGFP-C1 with the indicated reagents. The molar ratio PUMan:FuGENE and PUMan:DOPE was 1:1.

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