Iron Oxide Nanoparticles stabilized with Dendritic Polyglycerols as Selective MRI Contrast Agents

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Supporting Information

Experimental Details

General: Commercial chemicals were obtained from Acros Organics (Belgium), Baker, Berkel AHK, VWR, Güssing GmbH, or Sigma-Aldrich (Germany) in reagent grade, and used as received without further purification. Ultrapure water with a measured resistance of 18.2 MΩ·cm was obtained from a Millipore purification system (Milli-Q® Academic). In this work the standard cell medium Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS), penicillin (P, 100 units/mL) and streptomycin (S, 100 µg/mL) (DMEM/FCS/P/S) was used. DMEM was purchased from Sigma-Aldrich (product number D5523). It contains inorganic salts, several amino acids, D-glucose and vitamins. Fetal calf serum was purchased from Gibco (catalogue number 10270). The major component of fetal calf serum (FCS) is the protein bovine serum albumin (66,463 Da). A solution of P and S was purchased from Sigma Aldrich (product number P4458). Air or moisture sensitive reactions

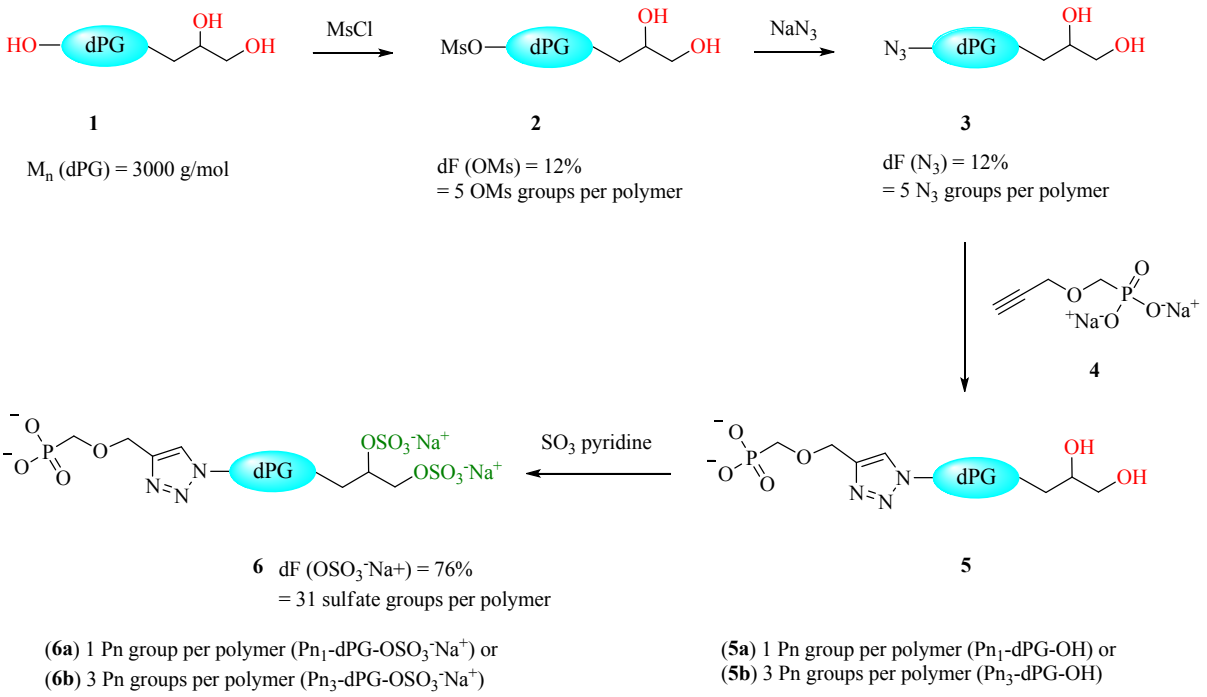
were performed in flame dried glassware under argon atmosphere or in the glovebox with oxygen and ultrapure water content of <1ppm. Ultrafiltration was performed in solvent-resistant stirred cells (Millipore, USA) with Ultracel regenerated cellulose membranes (MWCO = 1000 $g \cdot mol^{-1}$, Millipore, USA). Dialysis was performed in benzoylated cellulose dialysis tubings (MWCO = 2000 $g \cdot mol^{-1}$, Sigma Aldrich, USA).

Characterization of ligands and nanoparticles:

^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on an ECX400 of ECP500 spectrometer (Jeol, Japan) or on a Biospin 700 instrument (Bruker, Switzerland). IR spectra were recorded with a Nicolet AVATAR 320 FT-IR 5 SXC (Thermo Fischer Scientific, USA) with a DTGS detector from 4000 to 650 cm^{-1}. Absorption bands intensities are indicated as weak (w), medium (m), or strong (s). Elemental analysis was performed on a VARIO EL instrument (Elementar, Germany). The iron concentrations of iron oxide nanoparticles were determined by an atomic absorption spectrometer (AAnalyst 200, Perkin Elmer). Dynamic light scattering measurements were performed on a Delsa Nano C from Beckman Coulter. At least 5 individual light scattering measurements were performed for deriving the presented averaged values of cumulant fits. Before measuring the samples in ultrapure water, isotonic saline, and DMEM/FCS/P/S, the solutions were filtered through membrane filter (0.2 µm, PA, Carl-Roth, Karlsruhe, Germany). TEM images were recorded on a Zeiss EM CR-transmission electron microscope with 80 kV acceleration voltage. The samples were prepared by dipping 400 mesh copper grids coated by a ≈ 9 nm carbon film (Quantifoil) into a dispersion of the nanoparticle samples. The magnetic properties (T_1 and T_2 relaxation times) were measured on a mq40 NMR Analyzer (Minispec relaxometer) from Bruker at 0.94 T. The samples were measured in concentration ranges of 0.5 – 2.0 mM Fe in ultrapure water. Plotting the $1/T_1$-

and $1/T_2$-values as a function of the sample concentrations yielded the T_1-relaxivity R_1 and the T_2-relaxivity R_2, respectively.

Synthesis of the polyglycerol ligands: Dendritic polyglycerol (dPG) was prepared according to a literature procedure[1] with a molecular weight of M_n = 3000 g·mol^{-1}, a polydispersity index (M_n/M_w) < 1.7, and a degree of branching of ≈ 60% by ring opening multibranching polymerization of glycidol on a 1,1,1-trimethylol propane (TMP) starter. One gram dPG bears 13.5 mmol OH groups, accordingly dPG_{3kDa} bears ≈ 40 hydroxyl groups. The degree of functionalization was determined by NMR by integration of separated signals (e.g. mesyl or triazole) against the polymer scaffold and by elemental analysis. In the case of the azidation process, a quantitative conversion of the mesyl groups was assumed, that is in accordance with NMR and IR spectra. The number average molecular weights of all derivatized polymers were calculated from the respective conversions, as determined by ^{1}H NMR spectroscopy or from the sulfur content by elemental analysis. Dendritic polyglycerol was functionalized with one or three phosphonate anchors and subsequently with sulfate groups in a multi-step reaction (see Scheme S1).



Scheme S1: Synthesis of dendritic polyglycerol with one or three phosphonate binding sites (**5a** and **5b**) and subsequent conversion of its OH groups into sulfate groups (**6**).

*Dendritic polyglycerol mesylate (**2**):* A solution of dendritic polyglycerol (**1**, M_n = 3000 g·mol^{-1}, 7.0 g, 2.3 mmol, 94.5 mmol OH groups) in anhydrous pyridine (50 mL) was cooled to 0 °C. Mesyl chloride (1.10 mL, 1.60 g, 14.0 mmol, 6 eq.) was added over a period of one hour, the reaction was allowed to equilibrate to room temperature, and stirred for 24 h. Water (30 mL) was added and the mixture was stirred for another 30 min. The solvent was evaporated and the crude product purified by dialysis in methanol over two days (5x 500 mL). Evaporation of the solvent yielded the product as a yellowish gel (4.6 g, 1.4 mmol, 58%, M_n = 3475 g·mol^{-1}). Degree of functionalization: 12% (5 OMs groups per polymer). ^{1}H NMR (700 MHz, DMSO-d6): 5.30 – 4.85 (functionalized secondary dPG units), 4.85 – 4.35 (functionalized primary dPG groups), 3.80 – 3.19 (dPG backbone), 3.17 (C$\underline{H}_3$, OMs), 1.26 (s, C$\underline{H}_2$CH$_3$ starter), 0.80 (s, CH$_2$C$\underline{H}_3$ starter) ppm. ^{13}C NMR (176 MHz, DMSO-d6, δ): 80.5,

80.3, 80.2, 78.7, 78.3, 73.3, 73.0, 72.1, 71.9, 71.3, 71.2, 70.9, 69.9, 69.3, 69.1, 63.5, 61.5, 53.9, 49.1, 23.1, 22.7 ($\underline{C}H_2CH_3$ starter), 8.0 ($CH_2\underline{C}H_3$ starter) ppm.

*Dendritic polyglycerol azide (**3**):* Dendritic polyglycerol mesylate (**2**, M_n = 3475 g·mol^{-1}, 3.5 g, 1.0 mmol, 5.0 mmol OMs groups) was dissolved in anhydrous DMF (15 mL) and sodium azide (451 mg, 11 mmol, 4.5 eq. OMs group^{-1}) was added. The reaction was heated to 60 °C and stirred for 24 h. The solvent was removed and the crude product subjected to dialysis in ultrapure water/acetone (1:1) for 24 h. Evaporation of the solvent afforded the product as a slightly yellowish gel (2.4 g, 768 µmol, 76%, M_n = 3125·g mol^{-1}). Degree of functionalization: Quant. (Mesyl), 12% of the dPG functional groups (5 N_3 groups per polymer).^{1}H NMR (700 MHz, D_2O/DMSO-d6 (5:1), δ): 4.33 – 3.25 (dPG backbone), 1.38 (s, $C\underline{H}_2CH_3$ starter), 0.88 (s, $CH_2C\underline{H}_3$ starter) ppm. ^{13}C NMR (176 MHz, D_2O/DMSO-d6 (5:1), δ): 79.7, 79.4, 78.2, 77.9, 72.1, 70.9, 70.7, 70.4, 69.2, 69.0, 68.9, 62.6, 60.8, 53.10, 22.4, 22.0 $\underline{C}H_2CH_3$ starter), 6.9 ($CH_2\underline{C}H_3$ starter) ppm. IR (bulk) ν_{max}: 3356 (m, OH), 2871 (m), 2099 (m, N_3), 2037 (w), 1653 (w), 1456 (w), 1273 (m), 1066 (s), 930 (m), 868 (w), 661 (m) cm^{-1}.

*Sodium(prop-2-ynyloxy) methylphosphonate (**4**):* Phosphonate **4** was prepared according to a literature procedure in two steps by reacting commercial diethylhydroxymethyl phosphonate with sodium hydride and propargyl bromide followed by cleavage of the ethoxy groups with trimethylsilyl bromide and work up with aqueous NaOH.[1]

*Phosphonate functionalized dendritic polyglycerol (**5b** Pn_3-dPG-OH):* Dendritic polyglycerol azide (**3**, M_n = 3125 g·mol^{-1}, 525.0 mg, 0.17 mmol) was dissolved in a mixture of

tBuOH/ultrapure water (1:1, 24 mL) and at room temperature sodium(prop-2-ynyloxy) methylphosphonate (**4**, 102 mg, 525 mM, 3 eq.), prepared in two steps according to a previously reported procedure,[1, 2] copper sulfate (4.2 mg, 26.2 mmol, 5 mol-%), and sodium ascorbate (26.0 mg, 131 µmol, 25 mol-%) was added. The pH of the solution was adjusted to 8.5 by addition of a diluted sodium hydroxide solution and stirred for 24 h at room temperature. The solvent was evaporated and the crude product dissolved in a saturated EDTA solution and subjected to ultrafiltration (MWCO = 1000 $g \cdot mol^{-1}$) in ultrapure water. Evaporation of the solvent and freeze drying gave the title compound as a slightly yellowish viscous gel (521 mg, 145 µmol, 86%, M_n = 3600 $g \cdot mol^{-1}$). Degree of functionalization: Quant. conversion, 3 phosphonate groups per polymer (controlled by stoichiometry, quantified by NMR). Compound **5a** (Pn_1-dPG-OH) was prepared according to **5b**, however, using only one equivalent of phosphonate **4**. The analytical data are similar except for an additional azide band in the IR spectrum at 2100 cm^{-1}. ^{1}H NMR (400 MHz, D_2O, δ): 8.95 – 8.00 (m, 1 H, triazole), 4.67 – 4.40 (m, $C\underline{H}_2OCH_2P$), 4.32 – 3.18 (m, $C\underline{H}_2$-triazole, PG-backbone, $CH_2OC\underline{H}_2P$, $C\underline{H}_2O$-starter), 1.34 (s, $C\underline{H}_2CH_3$ starter), 0.84 (s, $CH_2C\underline{H}_3$ starter) ppm. ^{13}C NMR (101 MHz, D_2O, δ): 143.6 (N$\underline{C}$=), 126.2 (N$\underline{C}$H=), 79.5, 78.1, 72.2, 70.7, 70.4, 69.2, 68.9, 68.6, 64.9 ($CH_2O\underline{C}H_2P$), 62.6 ($\underline{C}H_2OCH_2P$), 60.8 ($C_{sec.}H$ triazole), 50.4 ($C_{prim.}H_2$ triazole) ppm. ^{31}P NMR (162 MHz, D_2O, δ): 17.5 ppm. IR (bulk) ν_{max}: 3353 (m, OH), 2872 (m), 1646 (w), 1456 (w), 1343 (m), 1058 (s), 931 (m), 850 (m) cm^{-1}.

*Phosphonate functionalized dendritic polyglycerol sulfate sodium salt (**6a** Pn_3-dPG-$OSO_3^-Na^+$):* Dry phosphonate functionalized dendritic polyglycerol (**5b**, M_n = 3600 $g \cdot mol^{-1}$, 191 mg, 53 µmol) was dissolved in dry DMF (2 mL) and heated to 60 °C. Separately, a solution of SO_3 pyridine (470 mg, 3.0 mmol, > 1eq. OH $group^{-1}$) in dry DMF (3 mL) was added dropwise

over a period of 4 h. The mixture was stirred for 1 d at 60 °C and subsequently quenched with ultrapure water. The pH value of the solution was immediately set to 9 with diluted NaOH and the solvent was evaporated. The product was dissolved in ultrapure water, filtered, and subjected to ultrafiltration (MWCO = 1000 $g \cdot mol^{-1}$) in ultrapure water. Freeze drying yielded the title compound as a slightly yellowish solid (287 mg, 40 µmol, 75%, M_n = 7250 $g \cdot mol^{-1}$). Compound **6a** (*Pn_1-dPG-$OSO_3^-Na^+$*) was prepared under identical reaction conditions. The analytical data are similar except for an additional azide band in the IR spectrum at 2100 cm^{-1}. ^{1}H NMR (400 MHz, D_2O, δ): 9.10 – 8.10 (m, 1 H, triazole), 4.70 – 4.55 (m, C$\underline{H_2}$OCH_2P), 4.43 – 3.20 (m, C$\underline{H_2}$-triazole, PG-backbone, CH_2OC$\underline{H_2}$P, C$\underline{H_2}$O-starter), 1.45 (s, C$\underline{H_2}$CH_3 starter), 0.92 (s, CH_2C$\underline{H_3}$ starter) ppm. ^{13}C NMR (176 MHz, D_2O, δ): 146.5, 145.6, 143.7 (N$\underline{C}$=), 128.2, 126.6 (N$\underline{C}$H=), 78.3, 77.3, 75.9, 71.0, 70.2, 69.5, 68.7, 68.3, 67.6, 67.0, 66.11 (d, $^1J_{CP}$ = 167 Hz, $\underline{C}H_2$P), 65.0 ($\underline{C}H_2$OCH_2P), 51.0 ($C_{prim.}H_2$ triazole), 22.0 ($\underline{C}H_2$CH_3 starter), 7.0 (C$H_2\underline{C}H_3$ starter) ppm. ^{31}P NMR (162 MHz, D_2O, δ): 16.4 ppm. IR (bulk) ν_{max}: 3471 (m, OH), 2933 (w), 2887 (w), 1642 (m), 1464 (m), 1355 (w), 1227 (s), 1111 (s), 1040 (s), 926 (s), 775 (s) cm^{-1}. Sulfur content from elemental analysis: 13.82%, dF (sulfate) = 76%.

Synthesis of iron oxide nanoparticles: Oleate functionalized iron oxide nanoparticles were synthesized (NP-OA) via a high temperature approach.[3] After the synthesis, the resulting nanoparticles were purified by washing with ethanol (5 times) and redispersed in chloroform to yield the final NP-OA stock dispersion with a concentration of 10±1 mg/mL NP-OA. This dispersion was stored at 4 °C under argon until used. In a typical ligand exchange procedure, the NP-OA dispersion (1 mL) was mixed with 10 mL chloroform and ultrasonificated for 20 min. This dispersion was stirred with 6 mL of ligand Pn_1-dPG-OH, Pn_3-dPG-OH or Pn_3-dPG-$OSO_3^-Na^+$ (6 mM) under argon for 24 h. In case of Pn_1-dPG-OH or Pn_3-dPG-OH, DMSO was

used as a solvent and in case of Pn_3-dPG-$OSO_3^-Na^+$ a mixture of DMSO:ultrapure water=3:2. Subsequently, the nanoparticles were sedimented with a magnet (1 T), purified by washing with ethyl acetate, dried under a flow of argon, redispersed in ultrapure water, and finally stored at 4 °C under argon.

Cell Experiments:

Cytotoxicity assay

HUVEC cells (PromoCell) were seeded in 96-well plates (5000 cells/well) and grown for 5 h. After adherence the medium was replaced against medium (200 µl/well) containing NP-Pn3-dPG-SO4 and NP-Pn3-dPG-OH (final conc. 0, 0.1, 1, 10, and 100 nM), respectively. After 48 h cytotoxicity was determined by the MTT-assay.[4]

Parallel plate flow chamber assay

The human cell line K562 stably transfected with L-selectin was used to study ligand binding in cell rolling assays. Cells were resuspended in Hanks' balanced salt solution (PAA, Pasching, Austria) to a final concentration of $1 \cdot 10^6$ cells/mL and transferred into a syringe. A parallel plate flow chamber (µ-slide VI, ibidi GmbH, Germany) was incubated with 30 µg/mL of the L-selectin ligand PSGL-1-Fc (R&D Systems, Wiesbaden, Germany) for 2 h at room temperature in order to coat the surface. Subsequently, the surface was blocked with bovine serum albumin (2 mg/ml) for 30 min. The flow channel was connected to a high precision perfusion pump KDS 101 (KD Scientific) and the assays were performed at a constant shear stress of 10^{-5} N/cm². For visualization of the cell rolling an inverted microscope IM (Carl Zeiss AG, Oberkochen, Germany) equipped with a digital camera AxioCam MRc (Carl Zeiss

AG, Jena, Germany) was used. To monitor inhibition of receptor-ligand interaction, cells were preincubated for 10 min at 37°C with NP-Pn_3-dPG-SO_4 at given concentrations. Movies were taken at 100× magnification for a 1 min period and the number of rolling cells (flux) was determined by counting.[5, 6]

L-selectin inhibition via flow chamber assay

Two representative movies (each 10 sec) are provided from the cell based flow chamber assay showing cell rolling on the ligand coated surface of the flow chamber (for details see Material and Methods). The field of view in these movies corresponds to 800 × 600 μm.

Movie S1 indicated as "untreated" refers to the experiments where the cells were not preincubated with the potential inhibitor. The number of rolling cells during a period of 1 min in such an experiment served as the reference and was set to 100% flux. Movie S2 indicated as "FeO-dPGS treated" corresponds to an analogously performed experiment in which the cells have been preincubated with a solution of 50 nM NP-Pn_3-dPG-$OSO_3^-Na^+$ for 10 min before they were injected into the flow chamber. Due to the binding of NP-Pn_3-dPG-$OSO_3^-Na^+$ to L-selectin expressed on the cell surface a strongly reduced cell rolling was observed which was quantified via counting of rolling cells and given as % flux in reference to the number of rolling cells determined from untreated cells (see Figure S1 below and Figure 3b in the Full Paper).

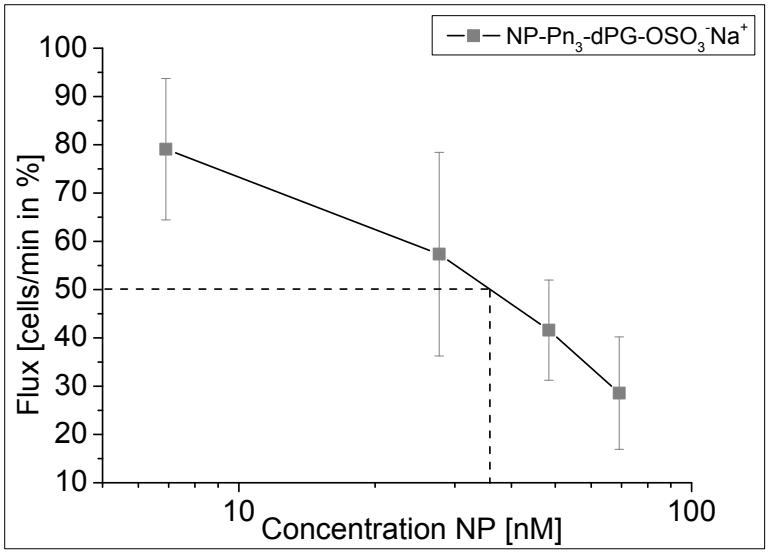


Figure S1: Competitive inhibition of L-selectin ligand binding by iron oxide nanoparticles functionalized with Pn_3-dPG-$OSO_3^-Na^+$ measured via a flow chamber assay.

SPR experiments

SPR experiments were carried out on a BIAcore X instrument (GE Healthcare) at 25 °C. A detailed description of the competitive selectin ligand binding assay has been published previously.[7] In brief, gold nanoparticles (15 nm diameter, Biotrend Chemikalien GmbH, Cologne, Germany) coated with L-selectin-IgG chimera (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) which mimic the leukocyte were passed over the surface of a SPR sensor chip corresponding to the vascular endothelium. This sensor chip presented a synthetic L-selectin model ligand (SiaLex-(20 mol-%)-PAA-sTyr-(5 mol-%)) (PAA = poly(acrylamide) (Lectinity Holdings, Inc., Moscow, Russia) on one lane and N-acetyl-lactosamine-PAA (Lectinity Holdings) on a second lane which serves as the reference. The iron oxide nanoparticles functionalized with Pn_3-dPG-OH and Pn_3-dPG-$OSO_3^-Na^+$ were dispersed in 0.020 M HEPES buffer (pH 7.4, with 0.150 M NaCl and 0.001 M $CaCl_2$) and a constant flow

rate of 20 μL min^{-1} was applied. The resulting binding signal in response units was corrected for the non-specific interaction which was simultaneously detected from the reference lane and was set to 100% L-selectin ligand binding and served as the positive control. To evaluate competitive binding of the functionalized iron oxide particles to L-selectin, a defined preincubation step with the L-selectin gold nanoparticles was performed before its passage over the sensor chip surface. Reduction of the binding signal relative to the inhibitor concentration was recorded and calculated in percent of the binding of the positive control. The inhibitor concentration that caused 50% reduction of binding was calculated and referred to as the IC_{50} value. Each concentration was applied at least in duplicate.

IR spectra

IR spectra of the ligand Pn_3-dPG-$OSO_3^-Na^+$ and iron oxide nanoparticles functionalized with this ligand (NP-Pn_3-dPG-$OSO_3^-Na^+$) are shown in Figure S2. The assignments of the peaks are given in Table S1.

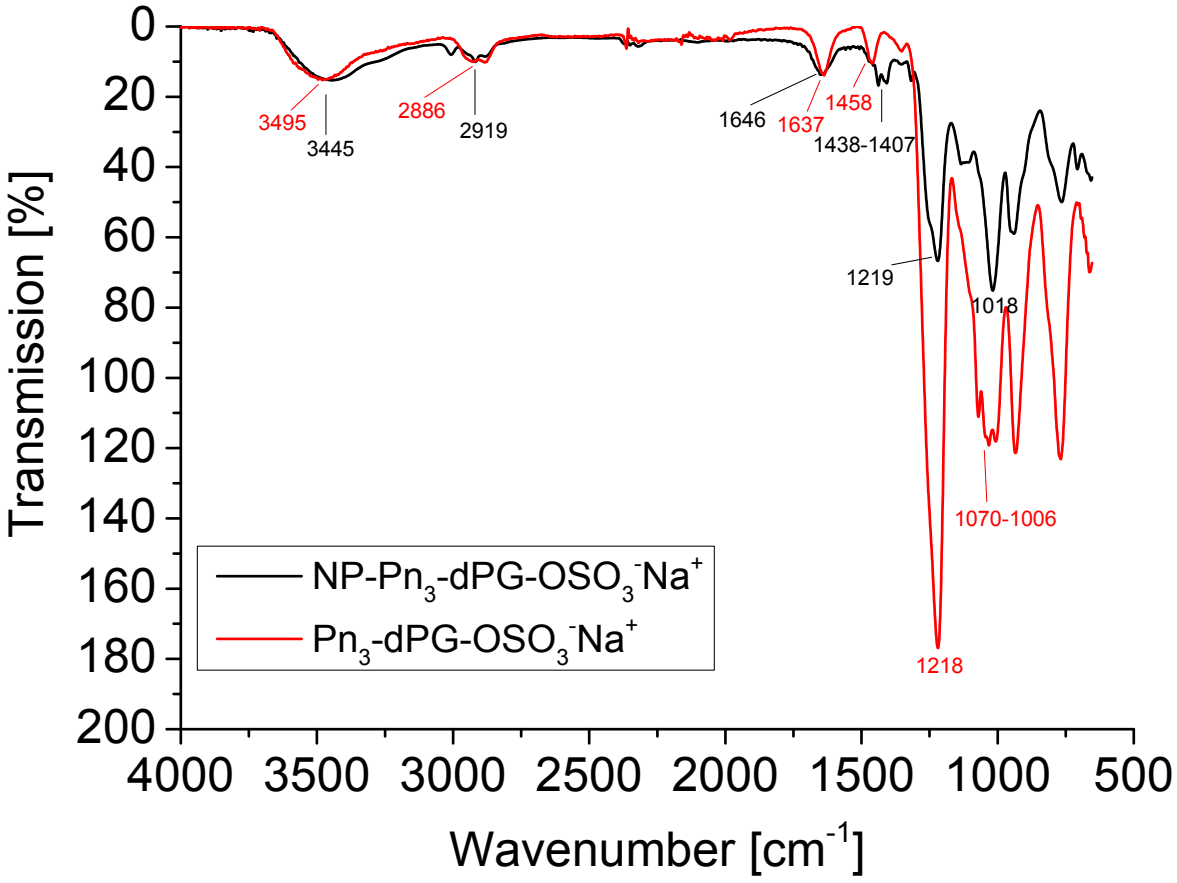


Figure S2: IR spectra of NP-Pn_3-dPG-$OSO_3^-Na^+$ and Pn_3-dPG-$OSO_3^-Na^+$

Table S1: IR-assignments for NP-Pn_3-dPG-$OSO_3^-Na^+$ and Pn_3-dPG-$OSO_3^-Na^+$

NP-Pn_3-dPG-$OSO_3^-Na^+$		Pn_3-dPG-$OSO_3^-Na^+$:	
Wavenumber [cm^{-1}]	IR band assignment (ν)	Wavenumber [cm^{-1}]	IR band assignment (ν)
3445 (m)	-OH in H-bond	3495 (m)	-OH in H-bond
2919 (w)	-CH_2-	2886 (w)	-CH_2-
1646 (m)	C=N-	1637 (m)	C=N-
1438-1407 (m)	P=O, R-SO_2-OR’	1458 (w)	(-CH_2-), m(R-SO_2-OR)
1219 (m)	P=O	1218 (s)	P=O, R-SO_2-OR
1018 (m)	Fe-O-P,S=O	1070-1008 (s)	PO_3^{2-}, S=O

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