

Design of safe nanotherapeutics for the excretion of excess systemic toxic iron

Srinivas Abbina,^{1,2,#} Usama Abbasi,^{1,2,#} Arshdeep Gill,^{2,3} Kendrew Wong,² Manu Thomas
Kalathottukaren,^{1,2} and Jayachandran N Kizhakkedathu^{1,2,3,*}

¹Department of Pathology and Laboratory Medicine, ²Center for Blood Research and Life Sciences Institute, ³Department of Chemistry, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

* Corresponding author

Email: jay@pathology.ubc.ca

Phone: 1-604-822-7085

Fax: 1-604-822-7742

These authors contributed equally to this work

Experimental section:

Materials and Methods

Materials. All the reagents, anhydrous solvents (dimethylsulfoxide, DMSO), and reagent grade solvents were purchased from Sigma-Aldrich, Canada, were used without further purification unless otherwise mentioned. Glycidol was purified by vacuum-distillation over CaH_2 at 40-50 °C and stored over 4 Å molecular sieves. Dialysis cassettes (MWCO 2 and 20 kDa) were purchased from Thermo Scientific (Rockford, IL, USA). Deuterated water and DMSO were purchased from Cambridge Isotope Laboratories, Inc. $\text{CH}_3\text{I}[3\text{H}]$ solution in toluene was purchased from ARC Radiochemical (St. Louis, MO) and further diluted in anhydrous DMSO and stored at -20 °C. Standard Regenerated Cellulose (RC) membrane (MWCO-50 and 10 kDa) was purchased from Spectrum, Inc., USA. The two biodegradable monomers, 2-(1-methyl-1-[2-(oxiran-2-yl methoxy)ethoxy]ethoxy) ethanol (DMK) (Scheme S1) and 2-(2-methyl-4-((oxiran-2-ylmethoxy)methyl)-1,3-dioxolan-2yl)ethanol (GHBK) and were synthesized using reported literature (Scheme S2).¹

Methods

NMR spectra (^1H , ^{13}C , and ^{13}C inverse-gated (IG)) of polymers were recorded on a Bruker Avance 300 and 400 MHz NMR spectrometers. Degree of branching of polymers was measured in deuterated water (D_2O) with a relaxation delay of 6 s. The degree of branching was calculated as per the reported procedure from the equation, $\text{DB} = 2\text{D}/(2\text{D} + \text{L})$, where D and L represent the intensities of the signals corresponding to the dendritic and linear units respectively). The absolute molecular weights of the polymers were determined by gel permeation chromatography (GPC) on a Waters 2695 separation module fitted with a DAWN HELEOS II multi angle laser light scattering (MALS) detector coupled with Optilab T-rEX refractive index detector, both from Wyatt Technology, Inc., Santa Barbara, CA. GPC analysis was performed using Waters ultrahydrogel columns (guard, linear and 120) and 0.1 N NaNO_3 buffer (pH = 8.5 and) was used a mobile phase, and dn/dc value for BHPG used is 0.12 mL/g. The hydrodynamic radii (R_h) of the polymers were obtained by quasi elastic light scattering (QELS) detector using a Wyatt Internal QELS instrument (angle of measurement, 99.9°, laser $\lambda = 620$ nm). Dynamic light scattering (DLS) measurements were made on Zetasizer instrument (NanoZS, Malvern Instruments). Absorbance readings for cell viability studies were measured at 570 nm on a SpectraMax 190 microplate reader from Molecular Devices. All the *in-vitro* testing of the iron chelating nanoconjugates was performed as per reported previously.² The activated partial thromboplastin time (aPTT) and prothrombin times (PT) of the chelators were measured by coagulation analyzer (ST4, Diagnostica Stago) using endpoint determination approach. Iron content in all tissues, feces, and urine was measured using inductive coupled plasma-mass spectroscopy (ICP-MS) on Agilent 7700 series ICP-MS. Fast prep tubes from MP biomedical were used for tissue homogenizer (BioSpec Product 96+, Bartlesville, OK). Nitric acid (1%) from VWR was used as matrix agent and indium was used as internal standard in analysis. Blood was collected from healthy unmedicated consented donors at the Centre for Blood Research; the protocol was approved by the University of British Columbia clinical ethics committee. All the animal studies were carried out at the Experimental Therapeutics laboratory, B.C. Cancer Research Centre, Vancouver, BC, Canada. All the protocols were approved by the Institutional Animal Care Committee (IACC), UBC.

Synthesis of biodegradable polymer scaffold

The synthetic protocol was followed from our previous publication.³ In briefly, BHPG-GHBK (86.4 kDa) was synthesized using the following the protocol (Scheme S2). A dried trimethylol propane (TMP) (25.0 mg, 0.186 mmol) was added to the three neck round bottom flask and stirred with potassium methylate (0.045 mL, 0.167 mmol) under argon for 30 min at RT. Excess methanol was removed under vacuum for 4-5 hr at 70 °C. Next, this flask was equipped with mechanical head stirrer and the reaction temperature was raised to 90 °C. A mixture of glycidol (1.50 mL, 0.0220 mol) and 2-(2-methyl-4-((oxiran-2-ylmethoxy)methyl)-1,3-dioxolan-2yl)ethanol (GHBKM)(4.80 g, 0.0220 mol) were added slowly using a syringe pump at a flow rate of 0.4 ml/hr and stirred it for 24 h (including monomer addition time) to form a core polymer with biodegradable linkages, followed by addition of glycidol (8.50 mL, 0.126 mol) alone at a flow rate of 0.4 mL/h. The reaction mixture was further stirred at same temperature for an additional 4 h. The resulting colorless sticky polymer was dissolved in methanol, precipitated three times from acetone to remove the unreacted monomers and dried under vacuum. The desired molecular weight is obtained without any fractional precipitation for the two attempts out of three (yield: 65 ± 5 %). Fractional precipitation was performed, by varying the volumes of MeOH and acetone, on third occasion to get the 86 kDa (1.3) BHPG-GHBK polymer. In brief, to a dissolved solution of BHPG-GHBK (1 g) in methanol (4 mL), acetone was slowly added (~ 1.5 mL) until the solution turned into cloudy. The resultant solution was centrifuged and precipitated polymer was isolated. The fraction contains very high molecular weight polymer. The supernatant was dried under vacuum and redissolved in methanol and the precipitation protocol was repeated. The volumes acetone should be optimized in each step according to formation precipitate in the solution. This process is repeated until the desired molecular weight of the polymer was obtained. The obtained polymer is dissolved in water and dialyzed against water for 24 h (MWCO-8 kDa, water replacements for every 8 h). The polymer solution was freeze-dried and stored at -20 °C.

The synthesis of the high molecular weight BHPG-DMKM and BHPG-GHBK polymers was performed using similar procedure by changing the monomer to initiator ratio.

The following protocol was used to synthesize the BHPG-GHBK (260 kDa): Initially, initiator TMP (12 mg, 0.092 mmol) was added to the flask and stirred with potassium methylate (0.045 mL) and a mixture of GHBKM (7.00 g, 32.1 mmol) and glycidol (2.16 g, 32.1 mmol) for 24 h to form a core polymer and the remaining glycidol (13.7 mL, 185 mmol) was subsequently added and the polymerization continued for additional 24 h to generate the high molecular weight BHPG-GHBK. Through the fractional precipitation, high molecular weight BHPG-GHBK (260 kDa, *D*-1.4) was isolated (yield - 3%) with approximately 15 % biodegradable content, and it was confirmed by ¹H NMR spectroscopy. The same protocol was repeated with DMKM monomer to synthesize BHPG-DMK (220 kDa, *D*-1.3). For BHPG-DMK polymer, fractional precipitation technique was used as mentioned above to get the desired molecular weight and dialysis was done against buffer (pH-9) to avoid polymer degradation (yield - 33%).

Synthesis of iron chelating nanoconjugates

The conjugation protocol was followed as reported in the literature.² The synthesis of a representative iron chelating nanoconjugate, BGD-60 with 60 DFO groups was listed here. BHPG-

GHBK (93 mg, 86 kDa, *D*-1.4, 0.001 mmol, 30% hydroxyl groups) was dissolved in water (15 mL) (buffer, pH-9 for BHPG-DMK-220 kDa) and NaIO₄ (64 mg, 0.299 mmol) was added and stirred for overnight. The resultant BHPG-GHBK-aldehyde was dialyzed against water (RC MWCO-8 kDa) for 24 h. Water was replaced three times. The number of aldehydes on BHPG were formed quantitatively and it was confirmed by UV-Vis spectroscopy. The BHPG-aldehyde was treated with 2,4-dinitrophenyl hydrazine in water or buffer and the resultant Schiff base of polymer will be precipitated out immediately, washed it gently with water or buffer. The precipitate was re-dissolved in DMSO (HPLC grade) and the absorbance maximum of the resultant Schiff base was used to determine to the number of aldehyde groups. Schiff base of formaldehyde and 2,4-dinitrophenyl hydrazine was used as a calibration standard. This polymer solution was used to load DFO on polymer. DFO.mesylate (63 mg, 1.5 eq, 0.097 mmol) was added to the resulting polymer solution, stirred for 24 h and treated with NaCNBH₃, (23 mg, 4 eq wrt to targeted DFO, 0.375 mmol), stirred for an additional 8 h. The unreacted aldehydes on BHPG-GHBK were quenched with excess ethanol amine (20 eq, 366 mg). The polymer solution was dialyzed against water/buffer for BDD-200, for 2 days (RC MWCO 8 kDa, water replacements for every 8 h). The conjugation of DFO on polymer was confirmed with ¹H NMR and number of DFO units on the polymer was confirmed UV-Vis spectroscopy ($\epsilon = 2300 \text{ Lmol}^{-1}\text{cm}^{-1}$). Size and molecular weight of the nanoconjugates were measured by GPC equipped with MALS detector. A complete size distribution profile of nanoconjugates was measured by DLS. DFO content on nanoconjugate was varied by treating the conjugate with different amounts of DFO. Briefly, the nanoconjugates (20 mg) were treated with Fe(II)SO₄ · 6H₂O (20 eq) at RT for 12 h and the reaction mixture was syringe filtered (0.2 μm). A small aliquot of filtered reaction mixture was used to measure the maximum absorbance of ferroxamine, a complex of DFO and Fe (III), at 540 nm in UV-Vis range, and used this absorbance to calculate the number of DFO units. The conjugate solution was stored at 4 °C. The polymer concentration was determined by TGA if it is needed.

***In vitro* degradation studies of iron chelating nanoconjugates:**

¹H NMR studies

Two nanoconjugates (BGD-60 and BDD-200) and pure biodegradable polymers, ~25 mg/mL in D₂O (pH 7.4), were transferred into NMR tubes and incubated at 37 °C for 10 days. The degradation kinetics of nanoconjugates was monitored by ¹H NMR.

GPC studies

Two nanoconjugates (BGD-60 and BDD-200, 10 mg/mL) were incubated in KCl/HCl buffer (pH-2.1) at 37 °C for 20 hr and neutralized to pH-7. The polymers solution was slightly concentrated on rotavap and dissolved in 1M NaNO₃ buffer, pH-8.5, loaded on GPC to analyze the degraded fragments of the nanoconjugates.

Dialysis based studies

Tritium labelled nanoconjugates (BGD-60 and BDD-200) and control (non-degradable HPG-DFO, 130 kDa), were incubated in KCl/HCl buffer (pH-2.1) at 37 °C for 20 h. The concentration of the degraded products along with undegraded conjugates was measured by scintillation counting before transferred into dialysis cassettes (MWCO-2 and 20 kDa). The concentration of the degraded products retained in the dialysis cassette was measured at different time periods to analyze the clearance profiles of nanoconjugates and compared with controls.

***In vitro* toxicity studies**

Cell viability of human hepatocellular carcinoma cells (HepG2 cell line, ATCC HB-8065) and mouse fibroblasts (NIH/3T3 cell line, ATCC CRL-1658) with different biodegradable iron chelating nanoconjugates at different concentrations were investigated by the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (ATCC 20-1010K) following manufacturer's protocol. In brief, cells were seeded at a density of 3×10^4 in a 96 well plate and maintained for an additional 3 days. Cells were treated with either DFO, deferiprone (DFP), deferasirox (DFX), nanoconjugates or media alone for 48 h at 37 °C, 5% CO₂. Following treatment, cells were rinsed with PBS three times, treated with MTT for 2 h followed by detergent-based lysis for 4-6 h. All readings were blanked. Following treatment, cells were rinsed with PBS three times and assay using the MTT assay. Cell viability was determined as follows [mean (570 nm) treated cells / mean (570 nm) untreated cells] x 100.

Hemoglobin oxidation assay

Protection against iron mediated oxidation of the hemoglobin for different biodegradable iron chelating conjugates was investigated by UV-Vis spectroscopy as described elsewhere.² Hemolysate was prepared from packed red blood cells (RBCs) and hemoglobin (HbA) concentration was determined according to Drabkin's method. Each sample contained 15.3 μ M HbA, 100 μ M Fe(III) of sulfate hydrate (Sigma Aldrich) and 1.25 mM of pre-chelated DFO equivalent (DFO or DFO conjugates). The level of oxidation was measured by spectrophotometric analysis (500-700 nm).

Determination of oxidative damage assay

Reactive oxygen species (ROS) were measured by 2',7'-dichlorofluorescein diacetate (DCFDA/H2DCFDA) Cellular Reactive Oxygen Species Detection Assay Kit (abcam 113851), as described elsewhere.^{4,5} HepG2 cells were grown in 24 well plates at a seeding density of 250,000 cells per well for 3 days. Then, cells were iron loaded with 400 μ M of iron from ferric ammonium citrate (Sigma-Aldrich, F5879) for 24 h. All cells were rinsed by PBS three times, after which they were treated with BGD-60, BDD-200, DFO, DFX, and DFP at 10 μ M chelator equivalent concentration for 24 h. To measure ROS, manufacturer's protocol was followed using the reagents in the kit. In brief, cells were washed once with 1x supplemented buffer, followed by a 45 minute incubation of 25 μ M of DCFDA (also in 1x supplemented buffer) at 37 °C. DCFDA containing buffer was replaced with 500 μ L of 1x supplemented buffer, and fluorescence was measured immediately 485 nm/535 nm (excitation/emission). This was done in duplicates and average arbitrary fluorescence units were plotted. Tertiary butyl hydrogen peroxide was used as positive control.

Blood coagulation measurements:

Activated partial thromboplastin time (aPTT) in human PPP

Polymer solutions (0.1 and 1 mg/mL were prepared in 20 mM HEPES buffer (pH 7.4 and 150 mM NaCl). Heparinized human PPP was prepared by mixing UFH solution and citrated human PPP. The final concentration of UFH in PPP was 4 IU/mL. The anticoagulant neutralization activity was examined by mixing 20 μ L of polymer solution with 180 μ L of heparinized plasma (1:10 v/v). The

final concentration of antidotes in plasma ranged from 12.5 to 250 µg/mL. 200 µL of aPTT reagent (Dade® Actin® FS Activated PTT, Siemens/Dade-Behring) was then added to the sample and 100 µL of this resulting mixture was transferred to cuvette-strips at 37 °C. The clotting time was measured on a STart®4 coagulometer (Diagnostica Stago, France). 20 mM HEPES buffer (pH 7.4 and 150 mM NaCl) added to heparinized plasma was used as a control for the experiments. All experiments were performed in triplicates and the average values (mean ± standard error of the mean) are reported.

Prothrombin Time (PT)

The final concentration of the iron chelating nanoconjugates in plasma was 1 and 0.1 mg/mL (9:1 v/v, PPP: conjugate). The stock solutions of the nanoconjugates were mixed with PPP and the coagulation cascade was initiated by adding a synthetic thromboplastin to the solution. All experiments were performed on a STart®4 coagulometer (Diagnostica Stago, France) in triplicates and the average values (mean ± standard error of the mean) are reported. HEPES buffer solution was used as a normal control.

Radiolabelling protocol of the macro chelators and controls

Radiolabelling of iron chelating nanoconjugates (BGD-60 and BDD-220) and control (HPG-DFO, 130 kDa) was performed as per our previous protocol.⁶ For labeling of BGD-60 with tritium the following procedure was used. Briefly, the buffered (pH-9) iron chelating nanoconjugate solution was replaced with DMSO by dialysing against DMSO for 24 h (DMSO replacements for two times) and stored over dried 4 Å MS. The polymer content (11 mg, 108400 Da, 100 nmol) in the solution was confirmed by TGA and the solution underwent three repeated freeze -thaw cycles to remove the dissolved oxygen. To the solution, another 5 mL of anhydrous DMSO was added under argon, followed by NaH (0.3 mg, 140 µmol). After stirring the solution at RT for 2 h, MeI-³[H] (100 µL) was added to methylate around 1 % of the hydroxyl groups. The solution was stirred for 20 h and 10 mL of phosphate buffer (pH 9) was added to quench unreacted NaH and the labeled polymer was purified by dialysis against phosphate buffer-pH 9 (RC MWCO 1000) until the dialyzate contained very low amounts of radioactivity (<100 dpm), for almost 48 h. The resultant labeled polymer solution was filtered through 0.2 µm syringe filter and the concentration (mg/mL) of the polymer was determined by weighing the dry polymer after freeze drying the known volume (50 µL) of the polymer. Activity of the tritium labelled nanoconjugates was measured by scintillation counter. The nanoconjugates solution in buffer pH-9 was used for animal studies by the addition of appropriate amount of NaCl in order to achieve the desired osmolarity and the specific activity was measured by scintillation counting.

Circulation time and organ biodistribution studies in mice

All the mice studies were performed at the Experimental Therapeutics Laboratory, British Columbia Cancer Research Centre, and Vancouver, Canada. The used protocol for animal studies was reviewed and approved by the Institutional Animal Care Committee (IACC) at University of British Columbia. Female BALB/c mice (6 – 8 weeks) were individually weighed and injected with nanoconjugates with a dose of 10 mg/kg of mice and 200 µl for 20 G mice. Mice were terminated by CO₂ inhalation at the end of the study (For BGD-60: 2, 4, 8, 24, 48, and 144 h, BDD-200: 1, 2, 4, 8, 24, and 48 h). Blood was collected by cardiac puncture with a 25G needle and placed into the appropriate microtainer tube. A 100 µL aliquot of whole blood was removed for

scintillation counting and the remaining volume processed for plasma. Hemolysis was minimized during the blood draw. Plasma was separated by centrifuging samples at 2500 rpm for 15 minutes, then pipetted off and placed into labelled vials shortly after collection. 50 μ L aliquot of plasma was measured for scintillation counting. Upon termination, the following organs were removed from mice, rinsed in PBS and weighed and processed for scintillation counting: whole liver, heart, spleen, lung, and both kidneys (in the same vial). Circulation half-lives and pharmacokinetic parameters of the conjugates were derived using two compartmental decay model in Origin 2018 software.

Urine and feces collection

For urine and feces collection, mice were housed in metabolic cages (144 h group) during the course of the study. Urine and feces were collected from the metabolic cages at 8, 24, 48, 72, and 144 h time points and placed in pre-weighed tubes. 100 μ L of urine was taken for scintillation counts. For feces collection, a 10% (or 5% if not significant amount) in a known amount of water using the Polytron tissue homogeniser and processed as per organs (below). 200 μ L aliquots of feces was read in triplicate by the liquid scintillation counter.

Processing of organs

Livers (whole) were made into a 30% homogenate in a known amount of water using a Polytron tissue homogeniser. Aliquots (in triplicate) of 200 μ L homogenate were transferred to scintillation vials. All the other organs and 100 μ L whole blood were taken directly into Solvable. Solvable® (500 μ l) was added to each vial to dissolve tissues. Vials were incubated at 50 °C overnight or up to a few days until completely dissolved, then cooled on ice prior to addition of 50 μ L 200 mM EDTA, 25 μ l 10 M HCl and 200 μ L 30% H₂O₂. This mixture was incubated at room temperature for one hour prior to addition of 5 mL scintillation cocktail. Samples were analysed by scintillation counting. Plasma was counted directly by addition of 5 mL scintillation cocktail to 50 μ L plasma. Triplicate aliquots (10 μ l) of stock tritiated compound were analysed by scintillation counting for specific activity determination.

Efficacy study of macro chelator in Fe/dextran iron overload mouse (female Balb/c) model

Mice were individually weighed and nanoconjugates and Fe-dextran (150 mg/kg) control were administered intravenously according to the body weights of mice. Four mice (N = 4) per group were used. The injection volume 200 μ L/20 G mouse was used to administer a dose of 100 mg of DFO/kg of mice. Following the intravenous administration of nanoconjugates, mice were returned to cages and continually monitored for acute signs of toxicity for the first two hours. Mice were monitored daily for acute signs of toxicity over a period of 21 days. Mice were iron overloaded with Fe-dextran on day-1 and nanoconjugates were injected in mice on day-8, 11, 14, and 17 and mice were terminated on day-21. Body weight was recorded prior to injection and three times per week. Mice were terminated by CO₂ asphyxiation according to the study grouping table. All animals were observed after post administration, and at least once a day, more if deemed necessary, during the pre-treatment and treatment periods for mortality and morbidity. In particular, no signs of ill health such as body weight loss, change in appetite, behavioural changes such as altered gait, lethargy, and gross manifestations of stress were noticed.

Following termination by CO₂ asphyxiation, 200 μ l whole blood in EDTA and 150 μ l serum were collected. Urine and feces were collected daily starting Day 8 (collection from Day 7 to Day 8) all

groups over the period of study and samples were stored at -80°C . ICP-MS was used to measure the elemental iron. Animals were terminated on Day 21 of experiment and entire organs (liver, heart, pancreas, kidney and spleen) were removed by standard procedure. For ICP-MS, organs were rinsed in PBS or saline, weighed, frozen in liquid nitrogen and stored at -80°C . For histological analysis, organs were rinsed in PBS or saline, and stored in 10% formalin until further processing.

Ferritin assay

Plasma ferritin level was assayed for each mice within the group using a quantitative colorimetric ELISA kit (Immunology Consultants Laboratory, Inc., Portland OR.) following manufacturer's protocol. In brief, samples were diluted accordingly and absorbance at 450 nm were analyzed using a 4-parameter logistic curve determined from standards.

Histological examination of liver and kidney

Histological analysis were performed on liver and kidney for all groups. 10% formalin fixed organs were parafilm embedded and sectioned. Both organs were stained for hematoxylin and eosin to stain the nucleus in blue, and the eosinophilic structures in pink. Liver sections were also stained for iron deposits using Prussian blue. All photomicrographs were captured on the Thermo Fisher's EVOS XL core imaging system, at a 20x magnification (a 0.868 $\mu\text{m}/\text{pixel}$ conversion factor).

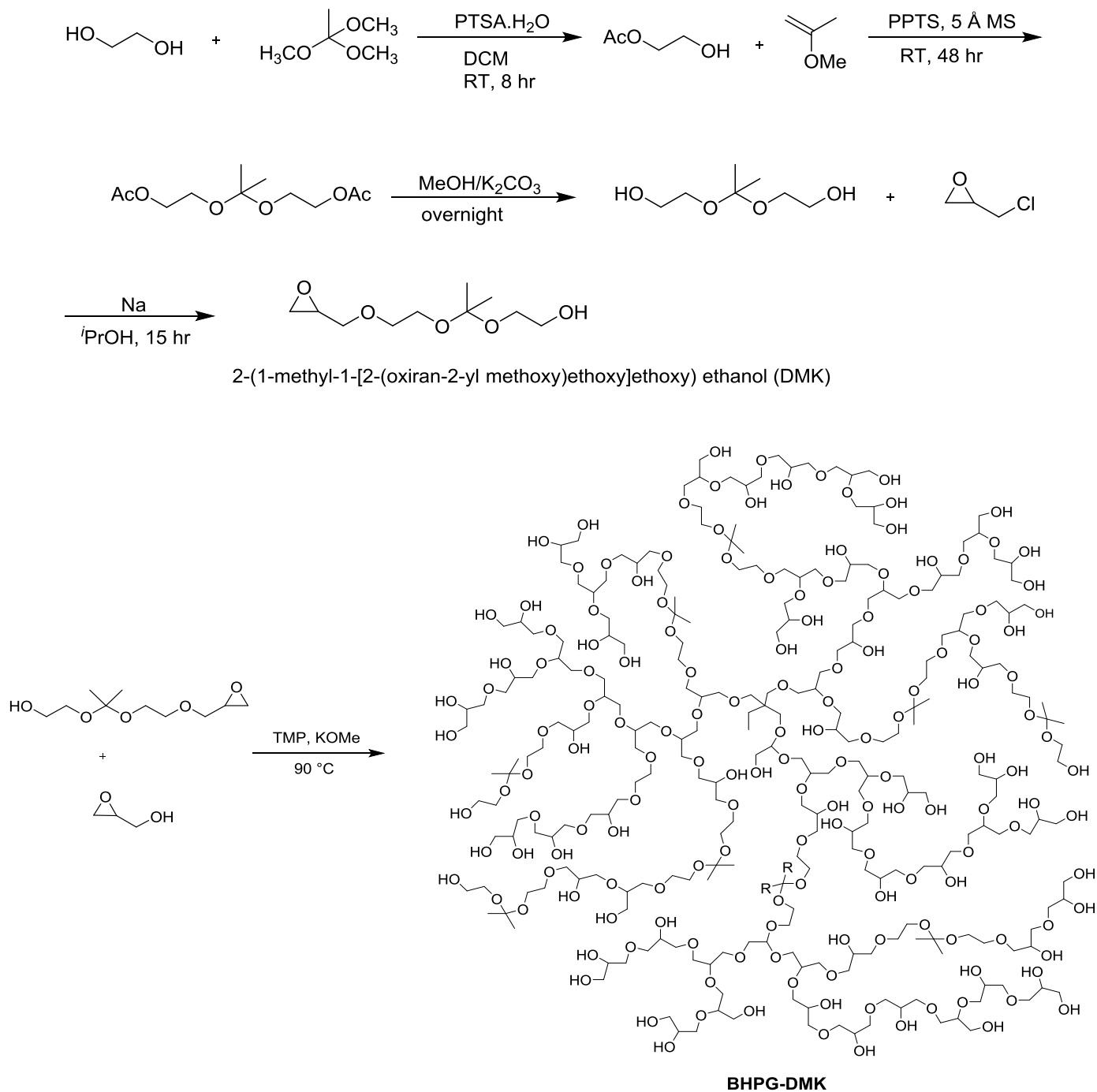
Quantification of iron levels by ICP-MS

Iron content in urine, feces, and organs (liver, heart, kidney, pancreas and spleen) were determined by inductively coupled plasma mass spectrometry (ICP-MS). Urine and feces were weighed in a vial and digested with concentrated nitric acid at room temperature overnight, followed by heating at 100°C until dried. Organs were sectioned, weighed in Fast-Prep tubes along with 0.5 mL of water, and homogenized for 5 minutes. Following homogenization, contents were transferred into vial and digested with concentrated nitric acid similar to urine and feces. All samples were completely digested through multiple rounds of hot nitric acid treatment. The final sample was prepared in 2% nitric acid and diluted accordingly for ICP-MS. Iron standards with different concentrations were also prepared in 2% nitric acid.

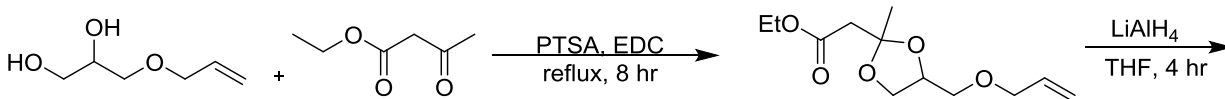
Statistical Analysis

Unpaired, two-tailed t tests were used for the statistical analysis of the data (Student t test with MS Excel using two samples with two equal variances method). Values were considered significant if $p < 0.05$. All data are presented as mean \pm standard deviation (SD) unless otherwise specified

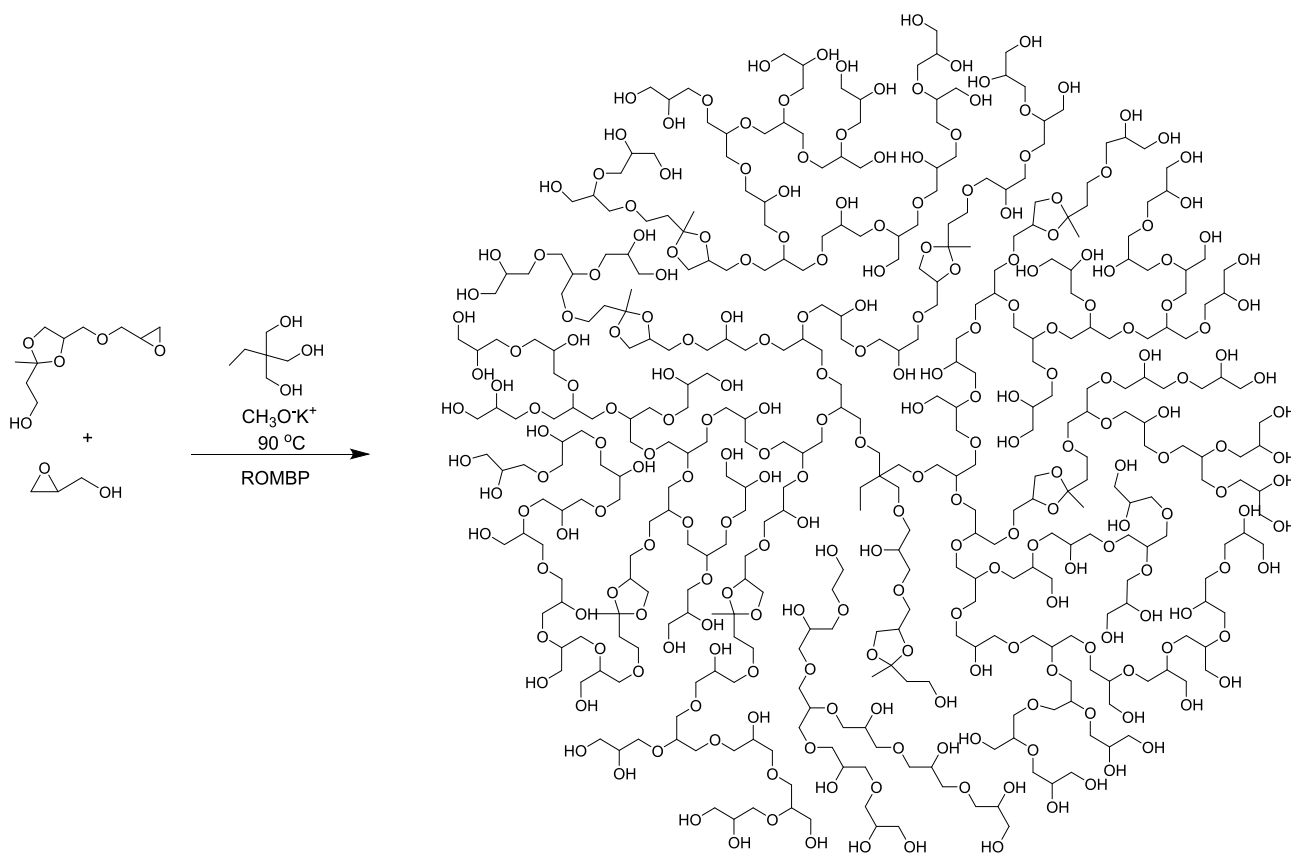
Supplemental Figures



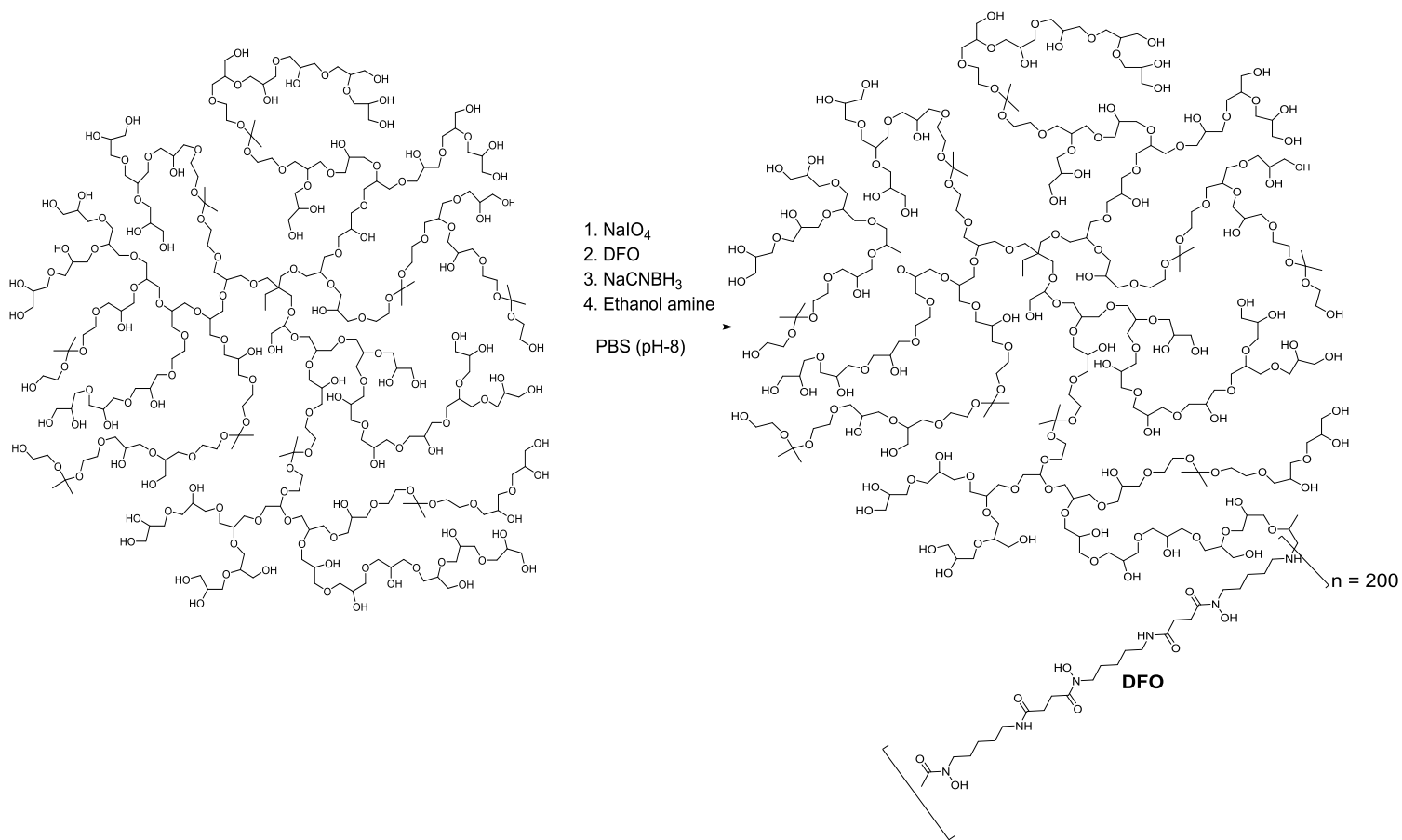
Scheme S1. Synthesis of DMK monomer and biodegradable hyperbranched polyglycerol (BHPG-DMK).



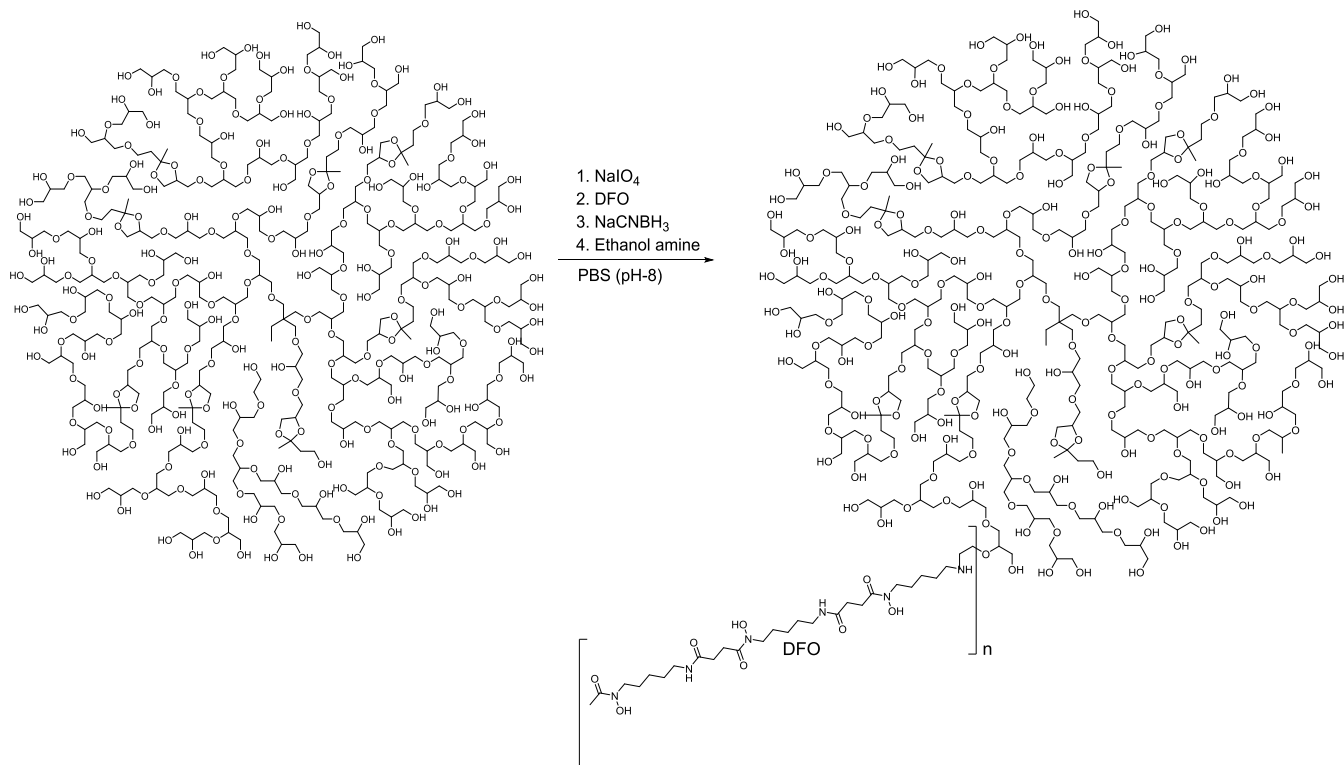
2-(2-methyl-4-((oxiran-2-ylmethoxy)methyl)-1,3-dioxolan-2yl)ethanol (GHBK)



Scheme S2. Synthesis of GHBK monomer and biodegradable hyperbranched polyglycerol (BHPG-GHBK).



Scheme S3. A representative synthetic scheme of BDD nano-conjugate.



Scheme S4. A representative synthetic scheme of BGD nano-conjugates ($n = 15, 30, 60, 110, 220$).

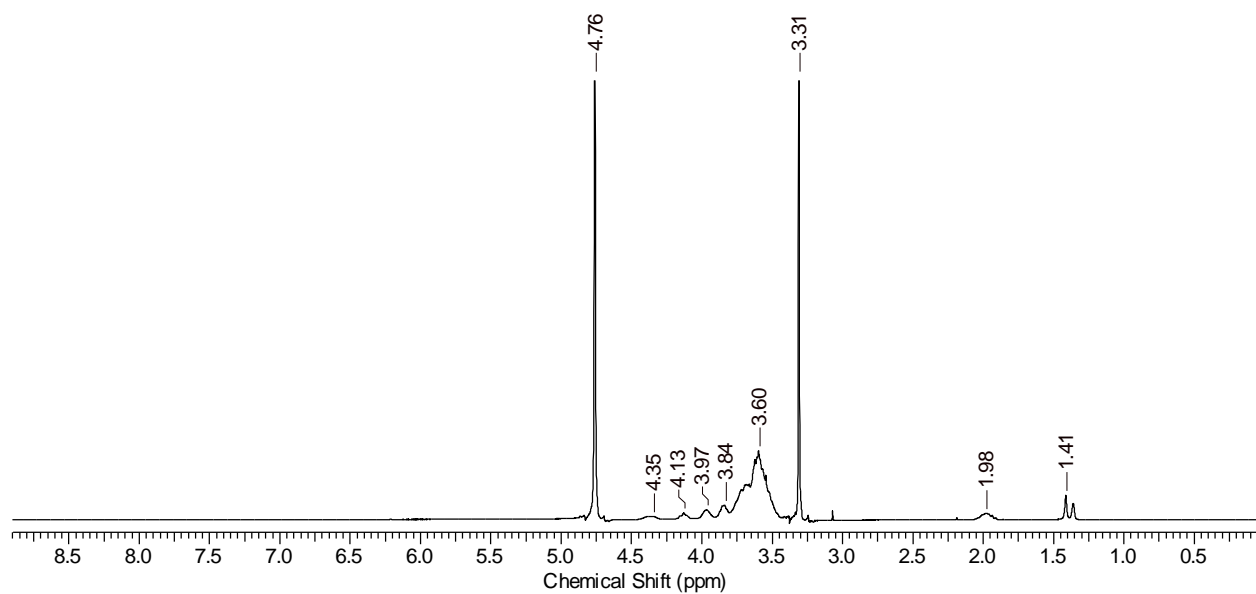


Figure S1. ¹H NMR spectrum of BHPG-GHBK (83 kDa)

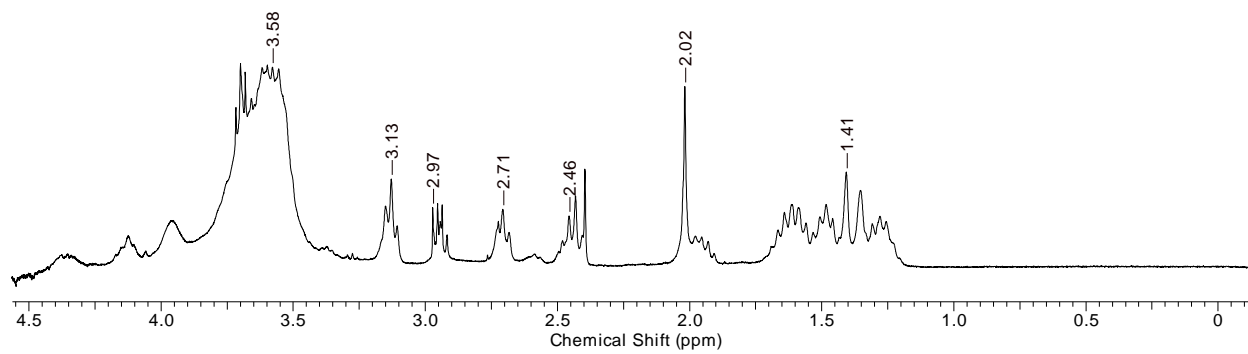


Figure S2. ¹H NMR spectrum of BGD-60

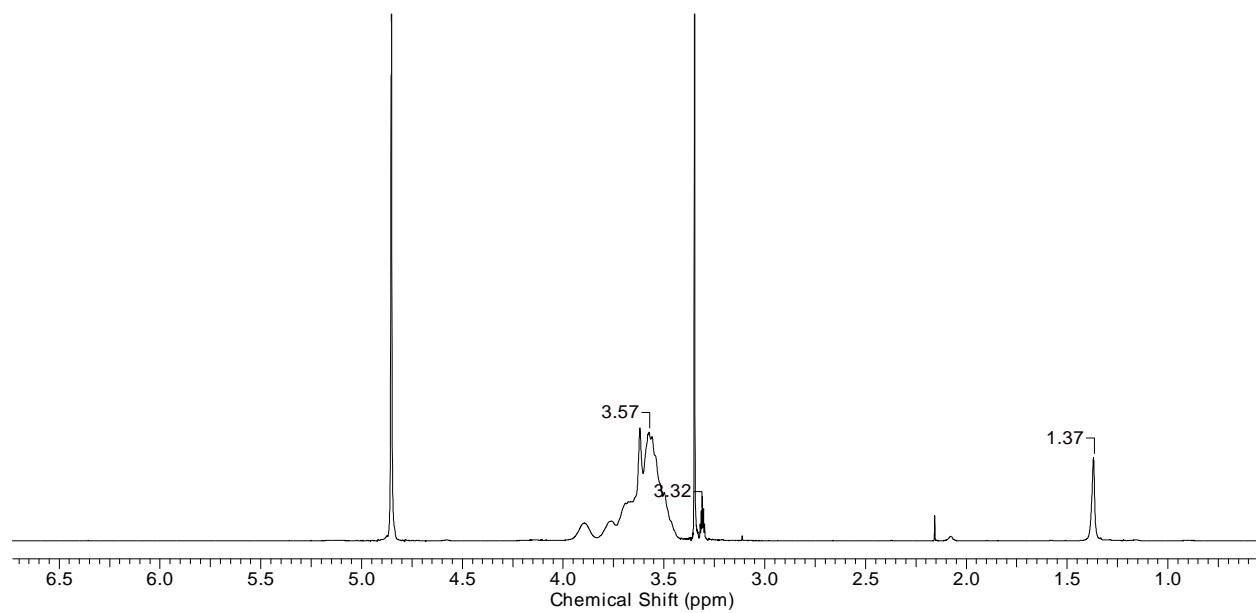


Figure S3. ¹H NMR spectrum of BHPG-DMK (220 kDa)

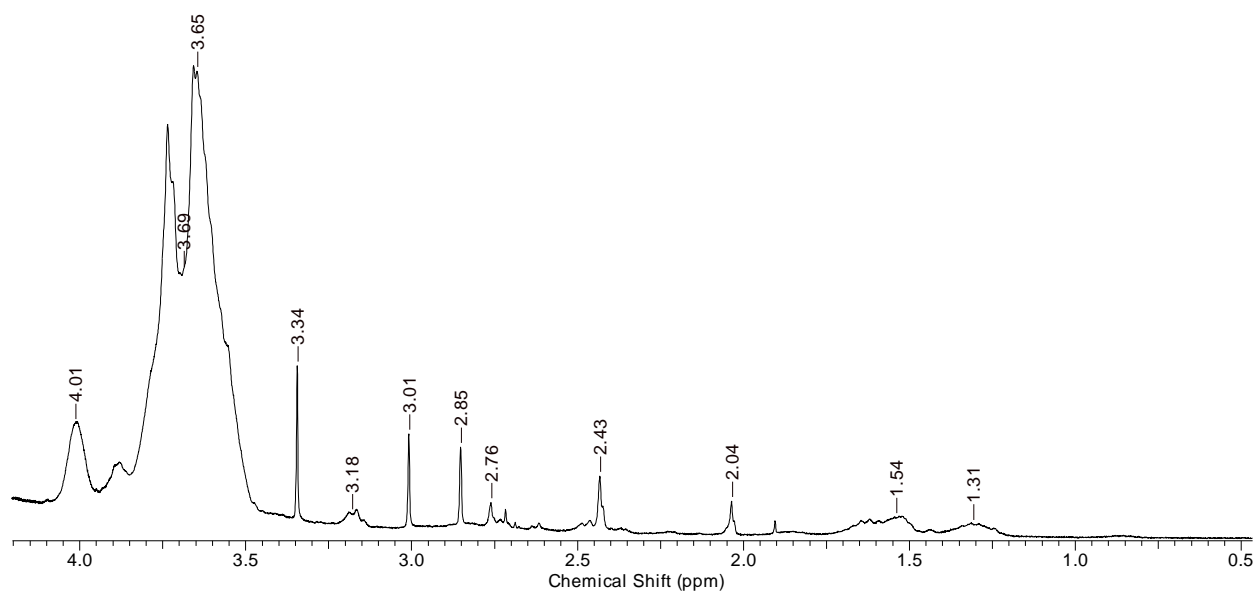


Figure S4. ¹H NMR spectrum of BDD-200

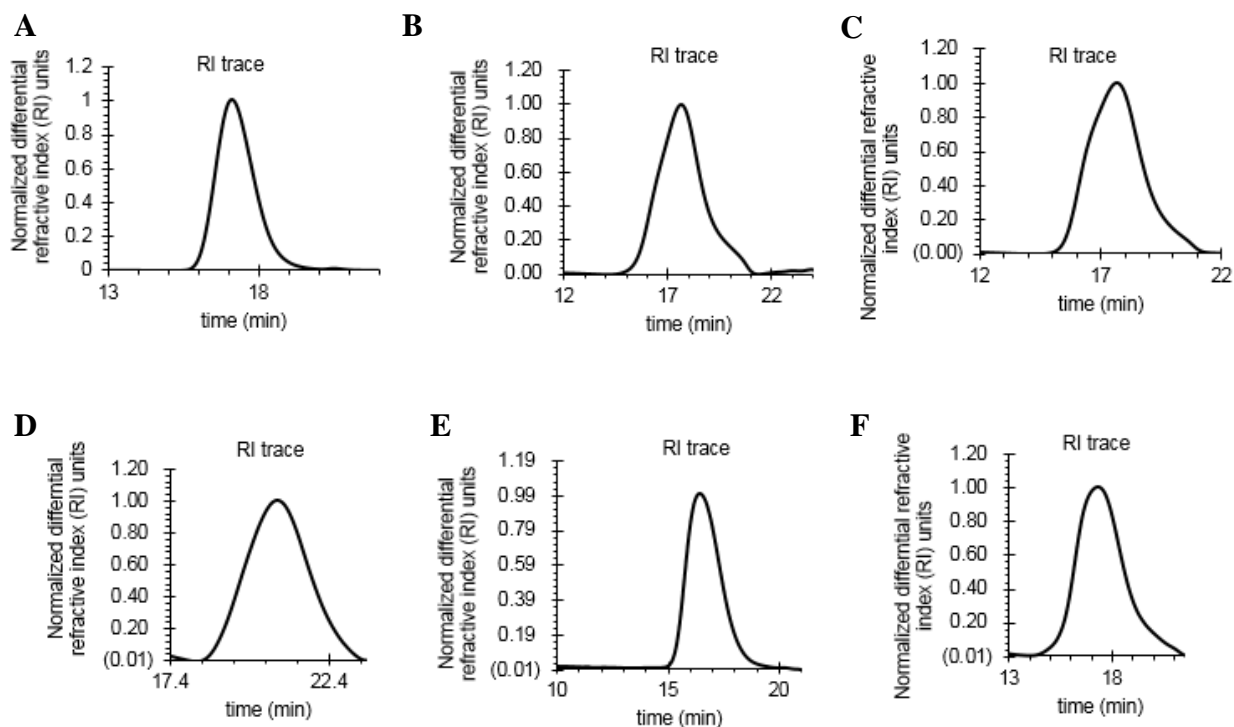


Figure S5. GPC traces of degradable polymers and nanoconjugates, (A) BHPG-GHBK (83 kDa), (B) BGD-60, (C) BHPG-GHBK (260 kDa), (D) BGD-220, (E) BHPG-DMK (220 kDa), and (F) BDD-200

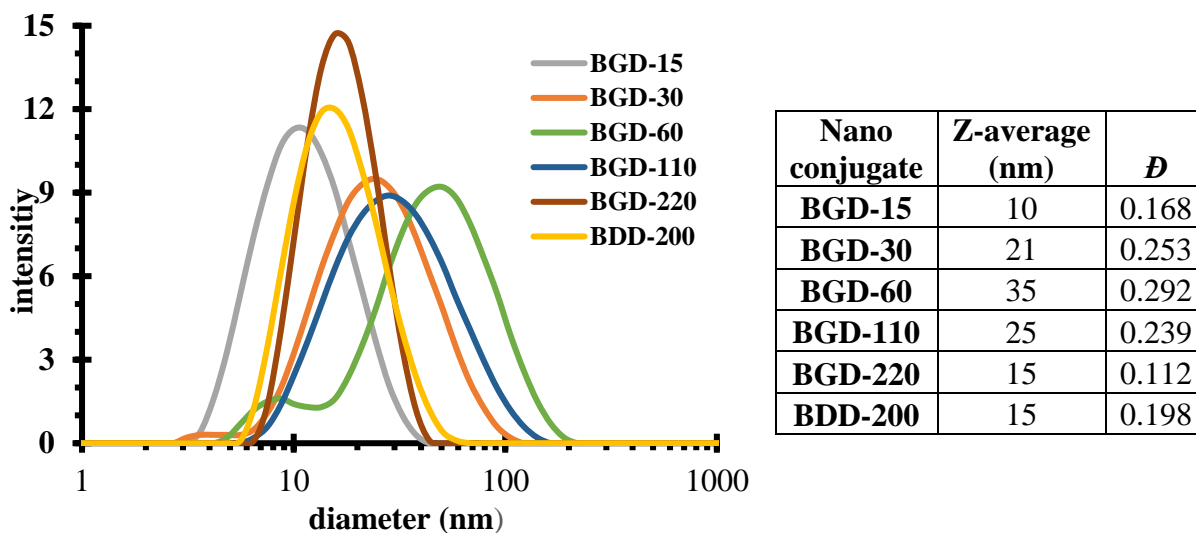


Figure S6. A full profile of DLS chromatograms of nanoconjugates. The size of the conjugates was reported in nm.

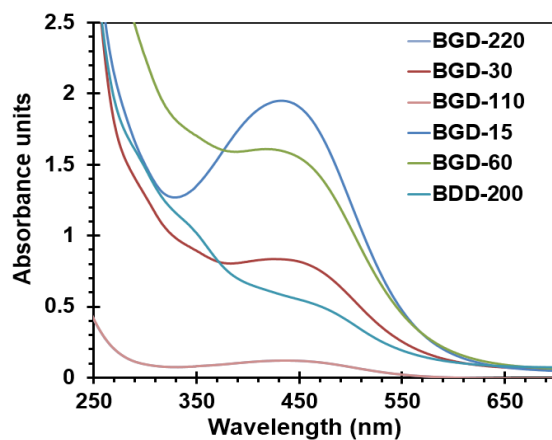


Figure S7. UV-Vis spectroscopy traces of nanoconjugates with DFO. The complexation of DFO with Fe(III) shows a characteristic peak at 540 nm, which was used to determine the number of DFO units on nanoconjugates.

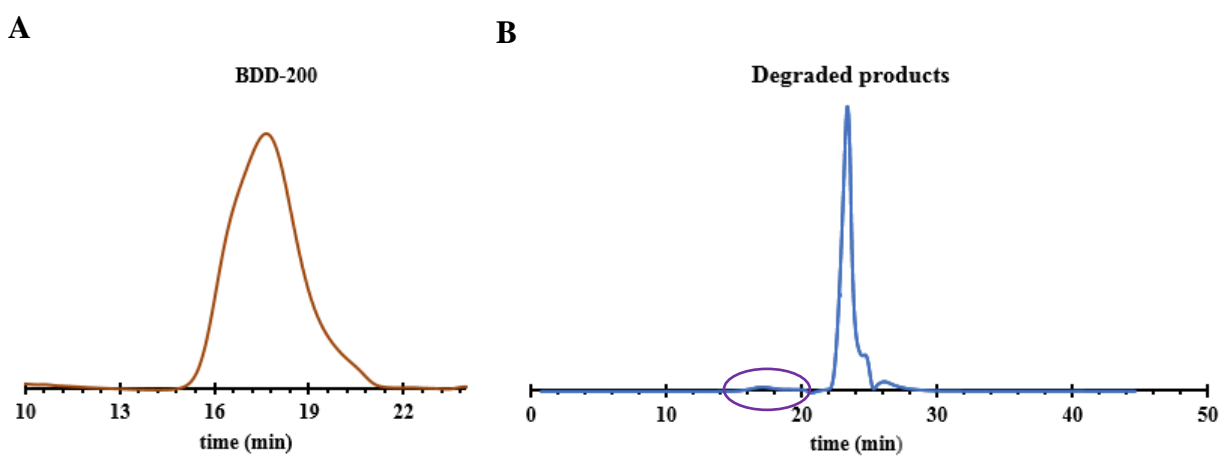


Figure S8. GPC traces of BDD-200 before (A) and after degradation (B). BDD-200 was incubated in KCl/HCl buffer (pH 2.1) for 20 h at 37 °C. The shift in chromatogram confirmed the degradation of BDD-200. The circled trace showed that less than 3% of the undegraded state of the BDD-200.

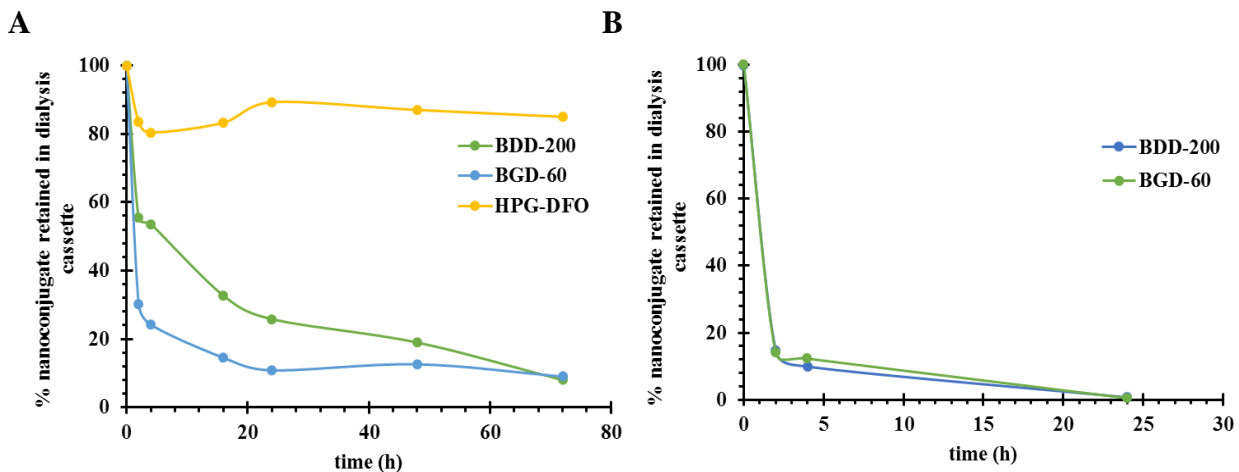


Figure S9. An *in vitro* diffusion-clearance profiles of degraded tritium labelled nanoconjugates through dialysis against water. (A) The degraded fragments from both nanoconjugates (BGD-60 and BDD-220) were slowly diffused from dialysis cassette with MWCO-2 kDa with in 72 h (> 90%) where as the non-degradable control (HPG-DFO) was retained upto 80% in dialysis cassette. (B) The degraded fragments were rapidly diffused (> 98%) from dialysis cassette with MWCO-20 kDa within 24 h. This study further confirmed that ketal linkages are crucial for degradation and clearance process of nanoconjugates.

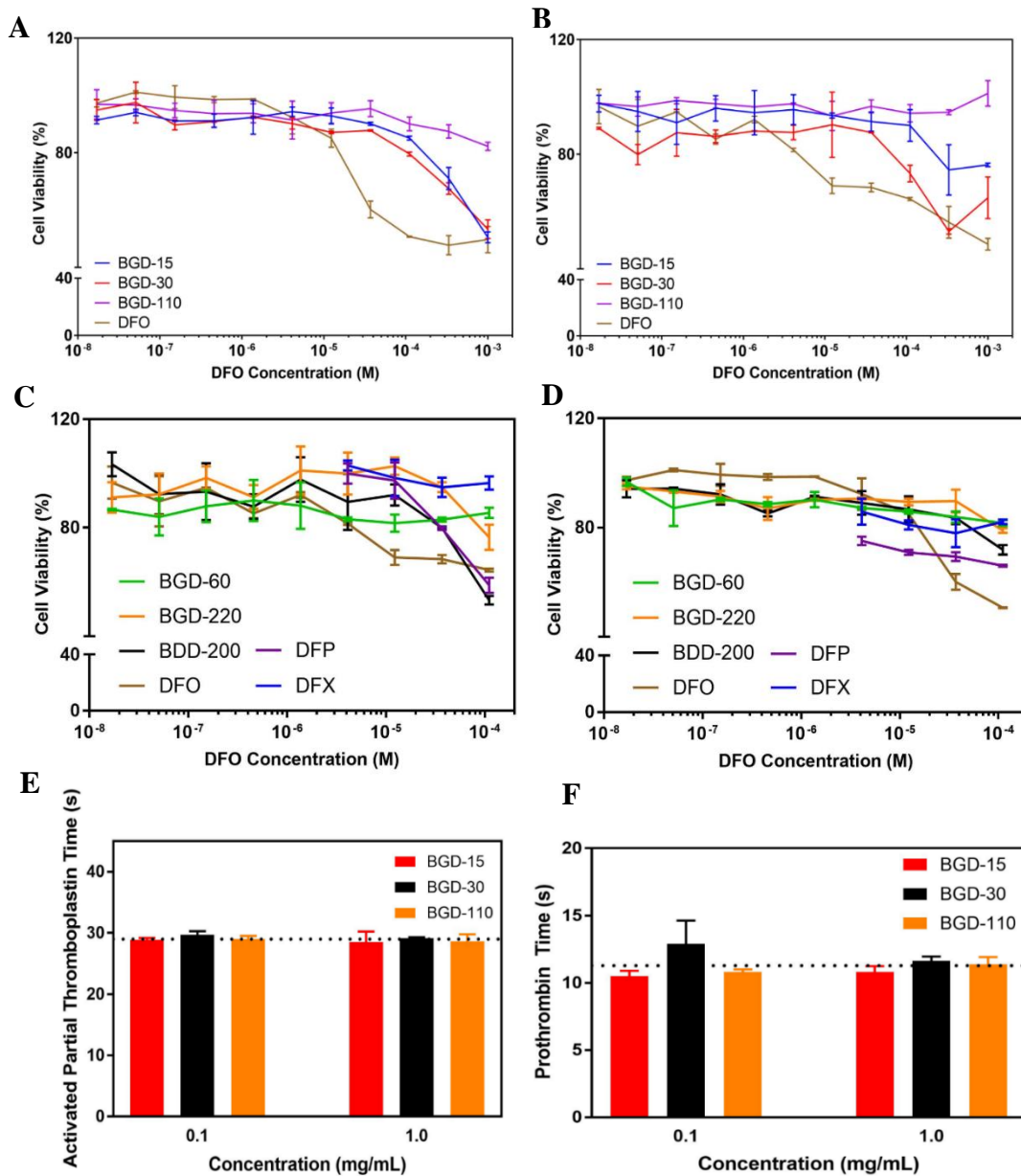


Figure S10. Cell viability studies of HepG2 (A&C) and fibroblasts (B&D) in the presence of nanoconjugates and controls (DFO, deferiprone (DFP), and deferasirox (DFX)). Cell viability was performed by incubating nanoconjugates and controls with both cell lines and cytotoxicity was measured after 48 h using the MTT assay. (E) Influence of macrochelators on activated partial thromboplastin time (aPTT) and (F) Prothrombin time (PT) at two different concentrations (0.1 mg/mL and 1 mg/mL).

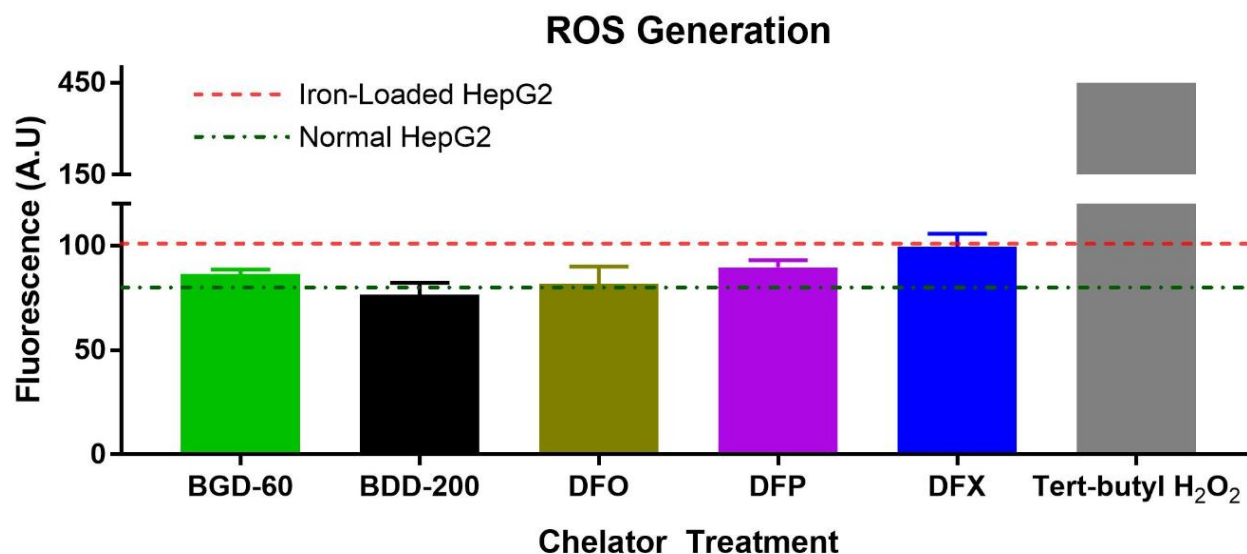


Figure S11. Cellular reactive oxygen species (ROS) generation detected in iron loaded HepG2 cells (ferric ammonium citrate, 400 μ M) and treated them with 10 μ M of Fe(III) specific chelators: nanocojugates at chelator equivalent concentration, deferoxamine (DFO), deferasirox (DFX), and deferiprone (DFP). The arbitrary fluorescence units represents the generation of ROS. Orange dotted line represents ROS levels in iron loaded, untreated HepG2 cells, and green dotted line represents ROS levels in unloaded, untreated HepG2 cells. While no significant differences were observed, nanoconjugates showed a reversal or decrease in ROS generation unlike DFX. Tertiary butyl hydrogen peroxide was used as a positive control.

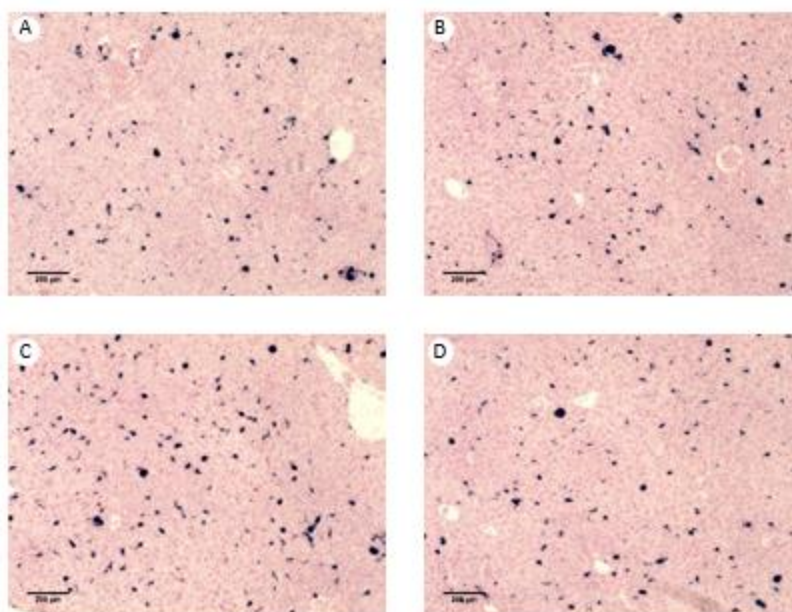


Figure S12. Histological examination of liver sections displaying tolerability and safety of nanoconjugates in iron overload Balb/c mice, stained for iron with Prussian blue, and a general hematoxylin and eosin (H&E) stain. All photomicrographs were captured at 20× magnifications from four treatment groups; panel (A) placebo control, (B) BDD-200, (C) BGD-60, and (D) DFO. Upon gross morphological examination, the plates of hepatocyte branch and anastomose maintain the normal sponge-like structure, liver sinusoids are undisturbed, and central veins are well defined. All treatment groups showed minimal necrosis or cellular injury.

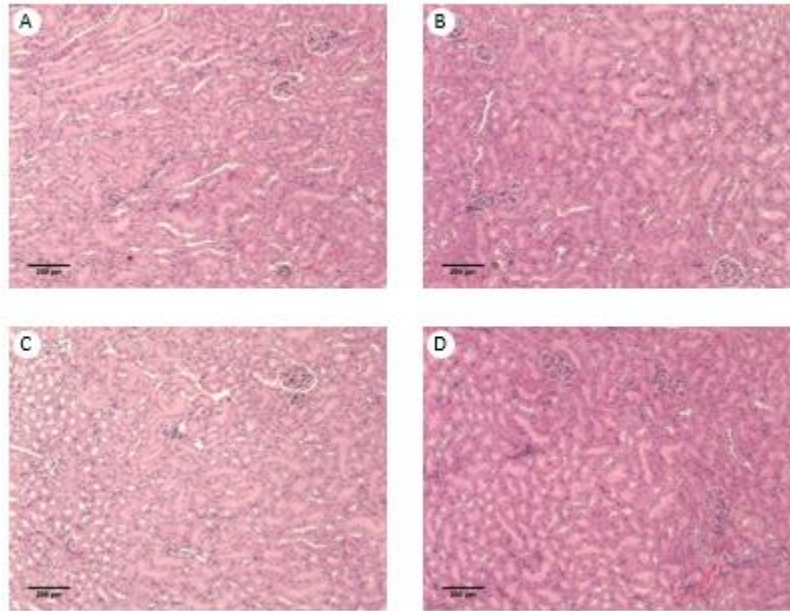


Figure S13. Histological examination of kidney sections display minimal damage upon treatment with chelators (nanoconjugates and DFO alone) in iron overload Balb/c mice. Photomicrographs are stained with H&E and captured at 20× magnifications for all four treatment groups; panel (A) placebo control, (B) BDD-200, (C) BGD-60, and (D) DFO. Gross examinations showed bowman's capsule is well maintained and defined, eosinophilic proximal convoluted tubules with smaller and less well-stained distal tubules. All treatment groups showed minimal necrosis or cellular injury.

organ	normal		Fe-dextran iron loaded		T-test	
	average Fe (ug) /organ (g)	SEM	average Fe (ug) /organ (g)	SEM	significance	P-value
liver	207.9	102.2	1250.0	67.8	yes	0.0001
heart	96.3	4.0	111.9	9.0	no	0.1630
pancreas	19.2	2.0	67.0	6.1	yes	0.0003
spleen	737.4	83.9	1666.0	88.5	yes	0.0003
kidney	72.5	5.4	126.0	9.2	yes	0.0024

Table S1. Quantification of iron levels in different organs from Fe-dextran treated (Fe-dextran iron loaded) and normal mice. No significant iron loading was observed in heart.

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