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PEGylated Functional Nanoparticles from a Reactive Homopolymer Scaffold Modified by Thiol Addition Chemistry

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ABSTRACT: Well-defined reactive polymer scaffolds are useful building blocks for a variety of biomedicine and nanotechnology applications. In this study, we have converted a RAFT-synthesized thiol-functional homopolymer scaffold (poly(pyridyl disulfide ethyl methacrylate), PPDSM) to poly(ethylene glycol) conjugated (PEGylated) nanoparticles via a straightforward approach. Poly(ethylene glycol) (PEG) was grafted to the reduced PPDSM via radical-mediated thiol−ene or Michael additions. The yield of PEG grafting via radical-mediated thiol−ene reaction and Michael addition was 68 ± 2 and 73 ± 1 mol %, respectively, of the total functional groups on the scaffold, as determined by ^{1}H NMR spectroscopy. The grafting yield via Michael addition reactions was non-linearly proportional to the reducing agent concentration used (thus the number of free thiols created on the polymer chain). It was observed by UV−vis spectroscopy that PEG grafting via Michael addition to the PPDSM takes place simultaneously with inter- and intrachain thiol−disulfide exchange reactions. Dynamic light scattering (DLS) measurements of PEG-acrylate (M_n = 2000 g/mol) grafted PPDSM (74 mol % grafting yield) in water showed the presence of particles with an average hydrodynamic diameter of 99 ± 8 nm and polydispersity index (PDI) of 0.22 ± 0.02. Atomic force microscopy (AFM) analysis of the same sample revealed the presence of spherical shape particles. ^{1}H NMR analysis of the same PEG grafted PPDSM nanoparticles in different solvents revealed that the PPDSM backbone in water was surrounded by PEG chains. Overall, the results indicate that simply grafting PEG to PPDSM homopolymer scaffold can be a straightforward route to the generation of nanoparticles with a biocompatible, stealth shell, and the present synthetic approach can be exploited further for the generation of PEGylated functional nanoparticles for potential drug delivery applications.

Introduction

Functional polymers having reactive pendant or terminal groups are of great interest for applications in biomedicine, nanotechnology, and biotechnology.[1−17] Thiol-functional polymers[2,18,19] are useful scaffolds as they allow transformations via various efficient and mild synthetic pathways (such as thiol−disulfide exchange,[20−23] oxidation,[2,20,24] thiol−ene addition[14,25−33]) suitable for numerous applications.

Previously, a functional polymer, poly(pyridyl disulfide ethyl methacrylate), PPDSM, having pendant pyridyl disulfide reactive groups, was synthesized by the reversible addition−fragmentation chain transfer polymerization (RAFT) (Scheme 1).[21] The development of new copolymers with controlled composition via single and simultaneous modifications of the RAFT-generated PPDSM homopolymer with free-thiol bearing small molecular weight compounds such as mercaptopropionic acid and a tripeptide was illustrated in a previous publication.[21] In this previous study, it was observed that tripeptide-modified copolymers generated from the PPDSM homopolymer scaffolds formed nanometer sized aggregates with spherical shape in water. This earlier result indicated the potential of PPDSM as a building block for the straightforward generation of nanoparticles for drug delivery applications. In other studies, amphiphilic block copolymers of PPDSM were also generated by copolymerization with biocompatible polymer blocks (i.e., poly(2-hydroxypropylmethacrylamide) and PEG) synthesized via RAFT polymerization and then used to generate reversibly cross-linked micelles and protein−polymer particles for potential drug delivery applications.[20,34]

In this current paper we describe the straightforward conversion of a PPDSM homopolymer scaffold to nanoparticles having a poly(ethylene glycol) (PEG) shell and residual functional pyridyl disulfide groups for further conjugation to bioactive agents. PEG, a water-soluble and nonimmunogenic polymer, is well-known for its stealth character. PEGylation is an effective strategy utilized to increase the blood circulation half-life of biomacromolecules and nanoparticles.[35−37] Thus, instead of synthesizing block copolymers of PPDSM and a biocompatible polymer with stealth character and then forming micelles/nanoparticles (as reported before[20,32]), grafting PEG directly to PPDSM scaffold should provide an alternative route to the generation of functional nanoparticles with a biocompatible, stealth shell.

In the present study, PEG chains were grafted to the reduced PPDSM via two different thiol addition pathways[14,25−33,38] (Scheme 1): (A) PEG having an acrylate functionality was grafted in the presence of a base via Michael addition and (B) PEG having an allyl group was grafted in the presence of a photoinitiator under UV radiation through radical addition. The reaction yields and the PEG grafting efficiencies via the two

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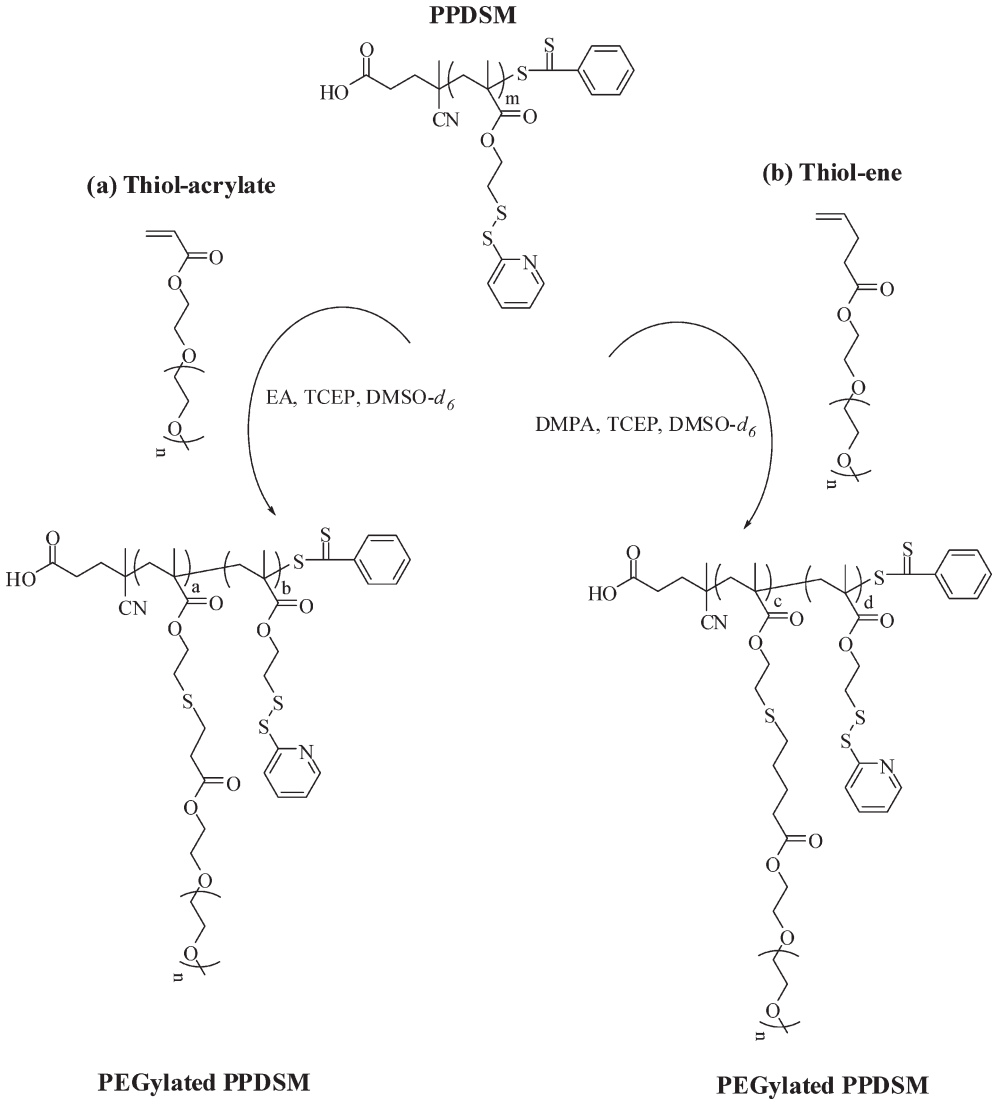
Scheme 1. PEGylation of PPDSM Scaffold via (A) Thiol−Acrylate and (B) Thiol−Ene Addition Reactions



different reaction pathways were determined using ^{1}H NMR spectroscopy. The effects of the reducing agent concentration and the PEG molecular weight on the grafting yield were investigated by a combination of ^{1}H NMR and UV−vis spectroscopy techniques. The formation of PEGylated particles in water was observed by ^{1}H NMR, dynamic light scattering, and atomic force microscopy techniques. The results indicate that the present synthetic approach can be exploited further for generating PEGylated nanoparticles from PPDSM homopolymer scaffolds for potential drug delivery applications.

Experimental Section

Materials. Poly(ethylene glycol) monomethyl ether (PEG monomethyl ether) (M_n = 2000 g/mol) and poly(ethylene glycol) (M_n = 10 000 g/mol) were purchased from Sigma-Aldrich. PEG (M_n = 2000 g/mol) was dissolved in toluene and subjected to rotary evaporation. This process was repeated a few times to remove residual water prior to use. 10 000 g/mol monomethoxy poly(ethylene glycol) was synthesized according an acylation reaction of one of the hydroxyl end-group of poly(ethylene glycol) (M_n = 10 000 g/mol) with acetyl chloride. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich) was neutralized with an equivalent number of moles of sodium hydroxide (NaOH) using a diluted solution and then freeze-dried. Ethylamine (EA) solution in methanol (2 M), 4-pentenoic anhydride (PA), 4-(dimethylamino)pyridine (DMAP), photoinitiator 2,2-dimethyl-2-phenylacetophenone (DMPA), PEG methyl ether acrylate (M_n = 454 g/mol) and 2-mercaptoethanol (ME) were purchased from Sigma-Aldrich and used without further purification. Deuterated chloroform ($CDCl_3$) with silver foil, deuterated dimethyl sulfoxide (DMSO-d_6), deuterated water (D_2O) and deuterated methanol (CD_3OD) were purchased from Cambridge Isotope Laboratories. Acryloyl chloride, stabilized with 400 ppm phenothiazine, was purchased from Alfa Aesar and freshly used to avoid a long storage period after opening. High-purity nitrogen (Linde gases, 99.99%) was used during polymer modifications. Poly(pyridyl disulfide ethyl methacrylate) (PPDSM; M_n = 9100 g/mol and PDI = 1.20 by GPC, Figures S1A and S1B, Supporting Information (SI)) was synthesized as reported previously.[21] Dichloromethane (DCM, Univar, analytical grade) was distilled over calcium hydride prior to use for macromonomer synthesis. *N*,*N*′-Dimethylformamide (DMF, Univar, analytical grade) was used for UV−vis measurements. *N*,*N*-Dimethylacetamide (DMAc) (CHROMASOLV plus, HPLC grade, Sigma-Aldrich) was used for polymerization and GPC analyses. Diethyl ether anhydrous (DEE, Univar) and HPLC grade methanol (MeOH, Univar) were used as received.

Instrumental Analyses. *^{1}H NMR Spectroscopy.* ^{1}H NMR spectra were recorded using Bruker DPX 300 and Bruker Avance III 400 instruments. Deuterated solvents, $CDCl_3$, DMSO-d_6, D_2O, and

CD_3OD were used for analyses. PPDSM structural analysis and both PEG-acrylate and PEG-allyl conjugation yields were determined via ^1H NMR analysis. PPDSM was analyzed in $CDCl_3$ (Figures S1A and S1B, SI). PEG grafting reactions were analyzed in DMSO-d_6 using a trace quantity of CD_3OD to dissolve neutralized TCEP. PEG-acrylate and PEG-allyl conjugation yields were characterized by comparing the integration of acrylate and allyl protons ($\delta \sim 5.8-6.4$ and 5.0−5.9 ppm, respectively, 3H) with the PEG characteristic protons.

Dynamic Light Scattering (*DLS*). Dynamic light scattering studies were performed using a Malvern Instruments Zetasizer NaNo ZS instrument equipped with a 4 mV He−Ne laser operating at $\lambda = 633$ nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple tau digital correlator electronics system. The polymer solutions were prepared in water at 2.5 mg/mL concentration.

UV−vis Spectroscopy. UV−vis spectra were recorded using a double-beam spectrophotometer (Cary 300) with a detection range from 190 to 800 nm and a photometric range of 5 absorbance units. The instrument was equipped with a 6×6 Peltier cell block, an accessory, and a temperature controller. The release of pyridine-2-thione (PT) was detected at 372 nm wavelength using DMF as a dilution solvent. A trace of water was used to dissolve TCEP before addition into the samples.

Atomic Force Microscope (*AFM*). A Nanoscope III (Digital Instruments, Santa Barbara, CA) AFM apparatus was used in tapping mode. 20 μL of the polymer solution (1 mg/mL) in distilled water was placed on a silicon substrate, and the sample was dried under atmospheric conditions at room temperature. The scans were typically done at rates between 1 and 4 Hz. The images were obtained using a silicon nitride cantilever with a nominal force constant of 0.38 N m^{-1}.

Methods. *Synthesis of PEG-acrylate*. PEG monomethyl ether (M_n = 2000 g/mol, 5 g, 2.5 mmol) was dissolved in distilled DCM (25 mL) in a 100 mL round-bottom flask. Triethylamine (TEA) (0.52 mL, 3.75 mmol) was added. Acryloyl chloride (0.303 mL, 3.75 mmol) was placed into a dripping funnel containing distilled DCM (15 mL). The solution was added dropwise into the PEG monomethyl ether solution maintained in an ice bath (at 0 °C), under vigorous stirring. The reaction mixture was stirred overnight in the dark. After taking a small amount of sample for the determination of the reaction yield by ^1H NMR, the crude reaction mixture was precipitated in diethyl ether (200 mL), dried under reduced pressure, and then dissolved in distilled water and purified using a dialysis membrane with a molecular weight cutoff (MWCO) of 1000 Da against methanol for a day and methanol: distilled water mixture for one more day. The acryloylation yield was 80 mol % of the PEG monomethyl ether feed as reactant, and PEG-acrylate purity in the final product was 75 mol % calculated from ^1H NMR analysis as indicated in Figures S2A and S2B in SI. The acryloylation yield was calculated considering the proportion of PEG reacted with acryloyl chloride to the total PEG present (ratio of D to all PEG characteristic peaks in Figure S2B where D represents the protons of reacted PEG, $-CH_2$ adjacent to ester formed upon reaction of PEG with acryloyl chloride). The PEG-acrylate purity was calculated considering the proportion of acrylate-functional PEG to the total PEG present (ratio of E to all PEG characteristic peaks in Figure S2B where E represents the protons of acrylate group in PEG-acrylate). The acryloylation yield and PEG-acrylate purity may be different if the double bonds of the acrylate-functional PEG are consumed partially, possibly due to polymerization, during synthesis and purification steps.

The same method was used to synthesize PEG-acrylate (M_n = 10 000 g/mol). The purity of this product was ~40%.

Synthesis of PEG-allyl. PEG monomethyl ether (M_n = 2000 g/mol, 5 g, 2.5 mmol) was dissolved in distilled DCM solvent (25 mL). Approximately 3 equiv of 4-pentenoic anhydride (1.37 mL, 7.5 mmol) was added into the PEG monomethyl ether solution. 0.15 equiv of 4-(dimethylamino)pyridine (DMAP) (46 mg, 0.38 mmol) was also added as a catalyst. The resultant solution was stirred overnight at 40 °C. The mixture was subjected to rotary evaporation to reduce the total volume to ~5 mL and then precipitated in anhydrous diethyl ether. The macromonomer was redissolved in DCM (5 mL) and reprecipitated in diethyl ether (200 mL). To maximize purity the precipitation procedure was repeated three times. The product, PEG-allyl, was dried under vacuum, then sealed under nitrogen gas, and stored in a freezer for further use. According to the ^1H NMR analysis of the final product, the esterification yield was 100 mol % of the PEG monomethyl ether feed as reactant and PEG-allyl purity in the final product was 74 mol % calculated from ^1H NMR analysis (Figure S2C, SI). The esterification yield was calculated by taking the proportion of PEG reacted with pentenoic anhydride to the total PEG present (ratio of F to all PEG characteristic peaks in Figure S2C where F represents the protons of esterification product, $-CH_2-CH_2$, adjacent to ester formed upon reaction of PEG with pentenoic anhydride). The PEG-allyl purity was calculated by taking the proportion of allyl-functional PEG to the total PEG present in the mixture (ratio of E to all PEG characteristic peaks in Figure S2C where E represents the protons of allyl in PEG-allyl). The esterification yield and PEG-allyl purity may differ if the double bonds of the allyl-functional PEG are consumed partially, possibly due to polymerization, during synthesis and purification steps.

PEG-acrylate and PEG-allyl Reactions with 2-Mercaptoethanol (*ME*). ME (0.305 mg, 3.9 μmol) was dissolved in DMSO-d_6 (200 μL). The solution was mixed separately with PEG-acrylate with M_n = 454 g/mol (purity of the acrylate ended PEG = 100 mol %, 2.043 mg, 4.5 μmol double bond; ME: acrylate mole ratio = 100:115), PEG-acrylate with M_n = 2000 g/mol (purity of the acrylate ended PEG = 75 mol %, 12 mg, 6.0 μmol PEG; 4.5 μmol double bond; ME: acrylate mole ratio = 100:115) or PEG-allyl with M_n = 2000 g/mol (purity of the allyl ended PEG = 74 mol %, 13 mg, 6.4 μmol PEG; 4.7 μmol double bond; ME: allyl mole ratio = 100:120) dissolved in 400 μL of DMSO-d_6. For thiol−acrylate reactions, ethylamine (3 mol % of ME, 0.12 μmol (0.0054 mg) in 8 μL in CD_3OD) was added into reaction solutions as a catalyst. It is worthwhile to note that the bases catalyze the addition of thiol to acrylate via Michael addition mechanism.[39] In accordance, most studies involving the thiol−ene Michael addition reaction have used ethylamine as a catalyst.[31,32] Alternatively, other primary or secondary amines[14,40−42] and phosphines[31,40,43] such as octylamine, hexylamine ethylenediamine, tris(2-carboxyethyl)phosphine, and dimethylphenylphosphine have been used as a catalyst in thiol−ene Michael additions. For thiol−ene radical reaction, DMPA (0.78 μmol (0.095 mg) in 50 μL of CD_3OD) was added into the reaction mixture. For all reaction solutions, TCEP (neutralized, 3.9 μmol (0.98 mg) in 30 μL of CD_3OD) was added dropwise into the reaction solution under a nitrogen atmosphere while stirring. The thiol−acrylate reactions were performed in the dark overnight. The next day, the solutions were analyzed by ^1H NMR to determine the reaction yields. The thiol−ene reaction was performed inside the UV-light hood at a wavelength of 365 nm for an hour. After the reaction, the solutions were analyzed by ^1H NMR to determine the reaction yields.

PEG-acrylate Grafting onto PPDSM. PPDSM (1 mg, 3.9 μmol PDS units, M_n = 9100 g/mol, PDI = 1.2 by GPC) was completely dissolved in DMSO-d_6 (200 μL). Here it should be noted that the number of PDS units was calculated by dividing the weight of the homopolymer by the formula weight of the PDSM monomer (255 g/mol). The solution was mixed separately with PEG-acrylate M_n = 2000 g/mol (12 mg, 6.0 μmol PEG dissolved in 400 μL of DMSO-d_6; 4.5 μmol double bond; purity of the acrylate ended PEG = 75 mol %) and PEG-acrylate M_n = 454 g/mol (2.043 mg, 4.5 μmol dissolved in 400 μL of

DMSO-d_6; 4.5 μmol double bond; purity of the acrylate ended PEG = 100 mol %). Ethylamine (3 mol % of PDS units, 0.12 μmol (0.0054 mg) in 8 μL of CD_3OD) was also added to catalyze the thiol−acrylate addition reactions. TCEP (neutralized, 3.9 (0.98 mg) μmol in 30 μL of CD_3OD) was added dropwise into the reaction solutions under a nitrogen atmosphere while stirring. The reactions were performed in the dark. The next day, the solutions were analyzed by ^{1}H NMR to determine the reaction yields. The crude mixtures were then dialyzed for 3 days using a membrane having a MWCO of 25 000 g/mol. Methanol and methanol:water mixture were used as solvents for the first and the second day of dialysis, respectively, followed by only water for the third day. Finally, the products were dried under vacuum at room temperature. The final products were analyzed by DLS and ^{1}H NMR.

In a separate experiment, PPDSM was reacted with PEG-acrylate (M_n = 2000 g/mol) in the presence of varying amounts of TCEP, following the same procedure outlined above. In these experiments, the PDS units:PEG-acrylate:EA:TCEP mole ratio was kept at 100:115:3:50 or 100:115:3:25.

PEG-acrylate (M_n = 2000 g/mol) grafting via radical addition reaction was also investigated (PDS units:PEG-acrylate: DMPA:TCEP mole ratio = 100:120:20:100 or 100:120:20:0) following the exact protocol described in the PEG-allyl grafting section.

PEG-allyl Grafting onto PPDSM. Both PPDSM (1 mg, 0.11 μmol polymer, or 3.9 μmol PDS units, M_n = 9100 g/mol, PDI = 1.2 by GPC) and PEG-allyl M_n = 2000 g/mol (purity of the allyl ended PEG = 74 mol %, 12.8 mg, 6.4 μmol of PEG; 4.7 μmol double bond) were prepared according to the procedure given in the PEG-acrylate grafting method. Then DMPA (0.78 μmol (0.095 mg) in 50 μL of CD_3OD) was added into the reaction mixture. TCEP (neutralized, 3.9 μmol (0.98 mg) in 30 μL of CD_3OD) was added dropwise into the reaction solution under a nitrogen atmosphere while stirring. The reaction was performed inside the UV-light hood at a wavelength of 365 nm for about an hour. After the reaction, the solution was analyzed by ^{1}H NMR to determine the reaction yield.

PDS Reduction by TCEP. PPDSM (0.8 mg, 0.09 μmol polymer, 3.12 μmol PDS units, M_n = 9100 g/mol, PDI = 1.2 by GPC) was completely dissolved in *N*,*N*′-dimethylformamide (DMF, 160 μL). The solution was mixed with PEG-acrylate (M_n = 2000 g/mol, 9.6 mg, 4.8 μmol dissolved in 320 μL of DMF, purity of the acrylate ended PEG = 75 mol %, 3.6 μmol double bond). Ethylamine (3 mol % of PDS units, 0.094 μmol (0.004 mg) in 6 μL of CD_3OD) was also added. The resultant solution was then equally divided into eight glass vials. TCEP (neutralized, in CD_3OD, equivalent to 20, 40, 60, and 80 mol % of PDS units) was added dropwise into the solutions under a nitrogen atmosphere while stirring. Each experiment was repeated twice. All the reaction solutions were added up to ~70 μL. The reactions were performed in the dark for 3 h. Half of the solutions was taken from each experiment and then diluted with DMF to 0.6 mL. The diluted solutions were then analyzed by UV−vis spectroscopy to determine the release of pyridine-2-thione (PT) with respect to the amount of TCEP added. The estimated concentration of PT release for 20, 40, 60, and 80 mol % TCEP addition with respect to 100 mol % PDS units were 65, 131, 196, and 261 μM, respectively. The concentration of PT released was calculated by using the equation $\varepsilon = A/bC$, where A is the absorbance obtained from a sample measurement, C is the estimated concentration of PT (M), and ε is the extinction coefficient of PT in DMF, i.e., $\varepsilon_{374\,nm}$ = 5440 M cm^{-1}, obtained from the calibration curve of standard PT solutions.[21]

Results and Discussion

In a previous study,[21] we showed that pendant pyridyl disulfide (PDS) groups of poly(pyridyl disulfide ethyl methacrylate) (PPDSM) can be reacted stoichiometrically with free-thiol compounds via disulfide−thiol exchange reactions. This provides an efficient synthetic route to the conversion of the homopolymer scaffold to random copolymers with new properties. Another potential of the PPDSM homopolymer scaffold, not investigated previously, is to exploit its high reactivity to ene-bearing molecules such as acrylate and allyl compounds. Upon reduction of PDS groups to free thiols, PPDSM should react easily with compounds having unsaturated double bonds via highly efficient addition reactions. It is well-known that thiol addition in the presence of a base catalyst can occur via a Michael addition mechanism involving hydrothiolation of unsaturated carbon−carbon double bond. In contrast, thiol addition initiated by radicals, e.g., using a photoinitiator under UV light, occurs through the formation of free thiol radicals that react instantaneously with carbon−carbon double bond.

In the present study, PDS groups along the PPDSM backbone were exploited via base-catalyzed thiol−acrylate and UV light-initiated thiol−ene reactions to convert the reactive homopolymer scaffold to an amphiphilic graft copolymer, which led to the formation of PEGylated, cross-linked nanoparticles in water. Modification of PPDSM via thiol−acrylate reactions was performed by grafting PEG-acrylates (M_n = 454 and 2000 g/mol) to the homopolymer in the presence of a catalytic amount of ethylamine (Scheme 1) while modification via thiol−ene reactions was performed by grafting PEG-allyl or PEG-acrylate (M_n = 2000 g/mol) using a photoinitiator (DMPA) under UV-light. The ^{1}H NMR spectra of PEG-acrylate (M_n = 2000 g/mol) and PEG-allyl (M_n = 2000 g/mol) used in the reactions are shown in Figures S2B and S2C of the SI. In all the reactions, PDS functional groups along the PPDSM chain were first reduced using a phosphine, tris(2-carboxyethyl)phosphine (TCEP), to yield free thiols for addition to the double bond of the PEG samples.

Thiol−Acrylate Reaction. A series of control experiments were first performed to optimize the thiol−acrylate reaction conditions. First, the stability of the acrylate bond in PEG-acrylate (M_n = 2000 g/mol) was investigated under the conditions to be used for the thiol−acrylate reactions. PEG-acrylate was incubated at room temperature overnight in the presence of the reagents to be used for the reaction, except TCEP (i.e., in the presence of PPDSM and ethylamine (EA)). The molar ratio of PDS units:PEG-acrylate:EA: TCEP was 100:115:3:0. It is noteworthy that generally thiol compounds are used in excess compared to the double bonds in both thiol−acrylate and thiol−ene reactions.[21] However, in our experiments, we intentionally kept the molar ratio of thiol compound less than that of the double bond to limit extensive intra- and intermolecular disulfide bond formation between the pendant groups of PPDSM chains. At the end of the incubation time of PEG-acrylate with PPDSM and EA, it was determined by ^{1}H NMR that 15 ± 5 mol % of acrylate bond of PEG-acrylate disappeared because of the polymerization reaction (Figure S4a in SI). In a subsequent control experiment, a low molecular weight thiol, 2-mercaptoethanol (ME), was reacted overnight at room temperature with two different PEG-acrylates (M_n = 454 and 2000 g/mol) in the presence of EA and TCEP using a ME:PEG-acrylate:EA: TCEP molar ratio of 100:115:3:100. If the addition reaction is successful, the ratio of the double bond protons of PEG-acrylate to the PEGs characteristics ethylene protons should decrease. Indeed, the disappearance of the double-bond of PEG-acrylate (signal E) upon the addition reaction could be easily monitored by ^{1}H NMR analysis (Figure 1A,B). As 15 mol % of PEG-acrylate undergoes unwanted polymerization under the reaction conditions, it was determined by ^{1}H NMR analysis that the yield of the reaction between ME and PEG-acrylates (M_n = 454 g/mol and M_n = 2000 g/mol)

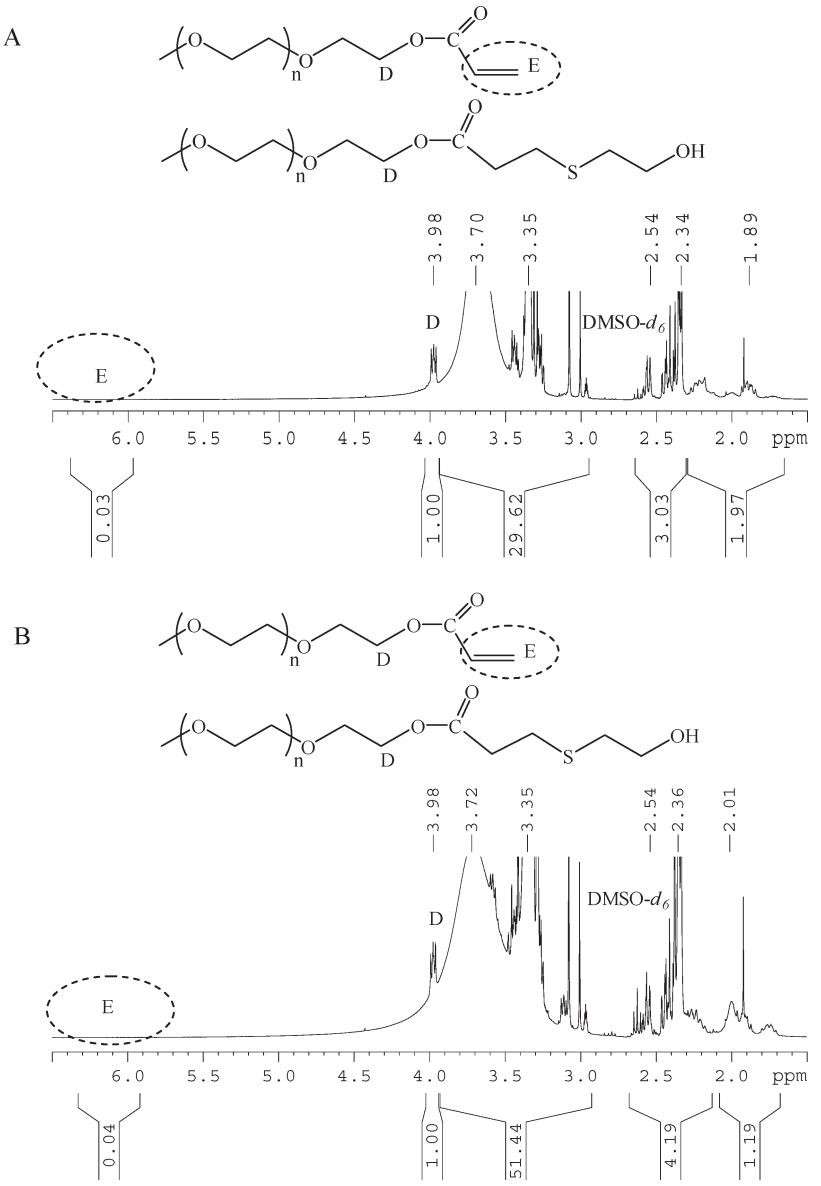


Figure 1. ^{1}H NMR spectra of the crude reaction mixture of 2-mercaptoethanol (ME) reaction with PEG-acrylate (A) M_n = 454 g/mol and (B) M_n = 2000 g/mol in $CDCl_3$. The reaction conditions: ME:PEG-acrylate:EA:TCEP molar ratio = 100:115:3:100, overnight, at room temperature. It should be noted that the crude reaction mixture contains nonreacted ME, TCEP, and ethylamine which cause signals overlapping with PEG signals at 3.7 and 3.3 ppm. The spectra show that 83 and 82 mol % of PEG-acrylate (M_n 454 and 2000 g/mol, respectively) in the feed reacted with ME (calculated by the following equation: $[(1 - ((I_E/3)/(I_D/2)) - 0.15) \times 100]$, where I_D and I_E refer to the integration values of signals D and E, respectively, and 15 mol % corresponds to the percent consumption of the double bonds due to the unwanted polymerization reaction under the conditions used. Considering that the feed molar ratio of PEG-acrylate:ME was 1.15, approximately 95 and 94 mol % of ME reacted with PEG-acrylate M_n = 454 and 2000 g/mol, respectively.

was 96 ± 1 and 95 ± 1 mol % of ME in the feed (average of two different experiments), respectively (Figure 1A,B). The high reaction yield proved that the conditions utilized were appropriate for an efficient thiol−acrylate addition.

When reduced PPDSM polymer was grafted with PEG-acrylate having M_n = 2000 g/mol, using the same reaction conditions to the control experiments outlined above, the grafting yield (defined as the grafted PEG percent with respect to total PDS units on PPDSM) was 73 ± 1 mol % (average of two different experiments), determined by ^{1}H NMR (Figure 2A) by comparing the signals of unreacted double bonds (signal E, 6.0−6.5 ppm) with the signal of $-CH_2$ adjacent to ester bond (signal D, at 4.3 ppm). The lower grafting yield relative to the reaction yield determined in the control experiment performed with ME and PEG-acrylate (M_n = 2000 g/mol) was attributed to the steric hindrance effect of the pendant thiol groups of PPDSM. It should be noted that increasing the PEG-acrylate (M_n = 2000) amount in the feed (PDS:PEG-acrylate:EA:TCEP molar ratio of 100:150:3:100) did not improve the grafting yield (data not shown).

In another set of experiments, PDS groups on the polymer were reduced to different extents (by varying the reducing agent concentration in the feed solution) and grafted with PEG-acrylate (M_n = 2000 g/mol) using the same reaction conditions (PDS units:PEG-acrylate:EA molar ratio = 100:115:3). The grafting yields were found to be nonlinearly proportional to the TCEP concentration (thus the number of free thiols created on the polymer chain). When TCEP was equivalent to 100, 50, and 25 mol % of the PDS units on the polymer, grafting yields were approximately 73 ± 1 mol %, 60 ± 1 mol %, and 36 ± 1 mol % (of the total PDS units on the polymer), respectively.

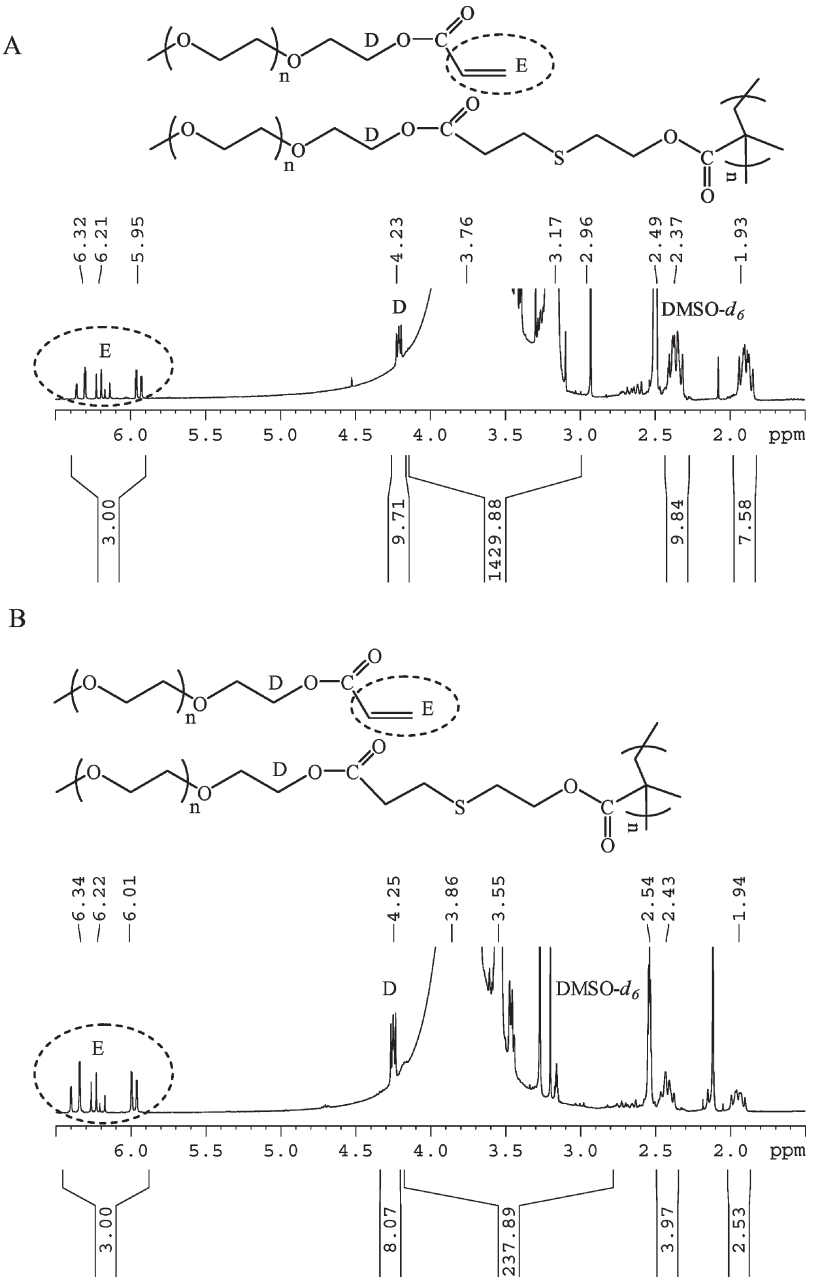


Figure 2. ^{1}H NMR spectra of the crude mixtures of PPDSM reaction with PEG-acrylate (A) M_n = 2000 g/mol and (B) M_n = 454 g/mol in DMSO-d_6. The reactions conditions: PDS units:PEG-acrylate:EA:TCEP molar ratio = 100:115:3:100, overnight, room temperature. It should be noted that the crude reaction mixture contains TCEP and ethylamine of which protons cause signals overlapping with PEG signals at 3.7 and 3.3 ppm. The signal of the residual water in the solvent also overlaps with the PEG signals. The spectra show that approximately 64 and 60 mol % of PEG-acrylates (M_n = 2000 and 454 g/mol, respectively) in the feed reacted with PPDSM (calculated by the following equation: $[(1 - ((I_E/3)/(I_D/2)) - 0.15) \times 100]$, where I_D and I_E refer to the integration values of signals D and E, respectively, and 15 mol % corresponds to the extent of the unwanted polymerization of double bonds. Considering that the feed molar ratio of PEG-acrylate:PDS units was 1.15, approximately 74 mol % and 69 mol % of PDS units grafted with PEG-acrylate (M_n = 2000 and 454 g/mol, respectively).

The ^{1}H NMR spectra of the crude mixtures of the reactions performed with varying TCEP ratios are presented in Figure 2A and in Figures S5a and S5b in the SI. While a yield slightly higher than the theoretical yield was observed at lower TCEP concentrations (targeting the reduction of 50 and 25 mol % of the PDS groups on the polymer), at the highest TCEP concentration (targeting the reduction of 100 mol % of the PDS groups on the polymer), addition of PEG-acrylate to the thiols on PPDSM occurred at a relatively lower yield. While the reasons for the former case are unknown, the relatively lower yield at the highest TCEP concentration was not surprising as at high TCEP concentration although more thiol groups become available for addition of PEG-acrylate, there is also an increased steric hindrance effect between PEG-acrylate macromolecules (M_n = 2000 g/mol) reacting with pendant thiol groups separated from each other by only a two carbon−carbon bond distance along the polymer chain. When a lower molecular weight PEG-acrylate (M_n = 454 g/mol) was reacted with PPDSM after reducing 100 mol % of PDS units, the grafting yield was 70 ± 1 mol % (Figure 2B), indicating that lower molecular weight PEG-acrylate still had a steric hindrance effect. This can be explained by the fact that highly hydrophilic PEG chains should possess a large hydrodynamic

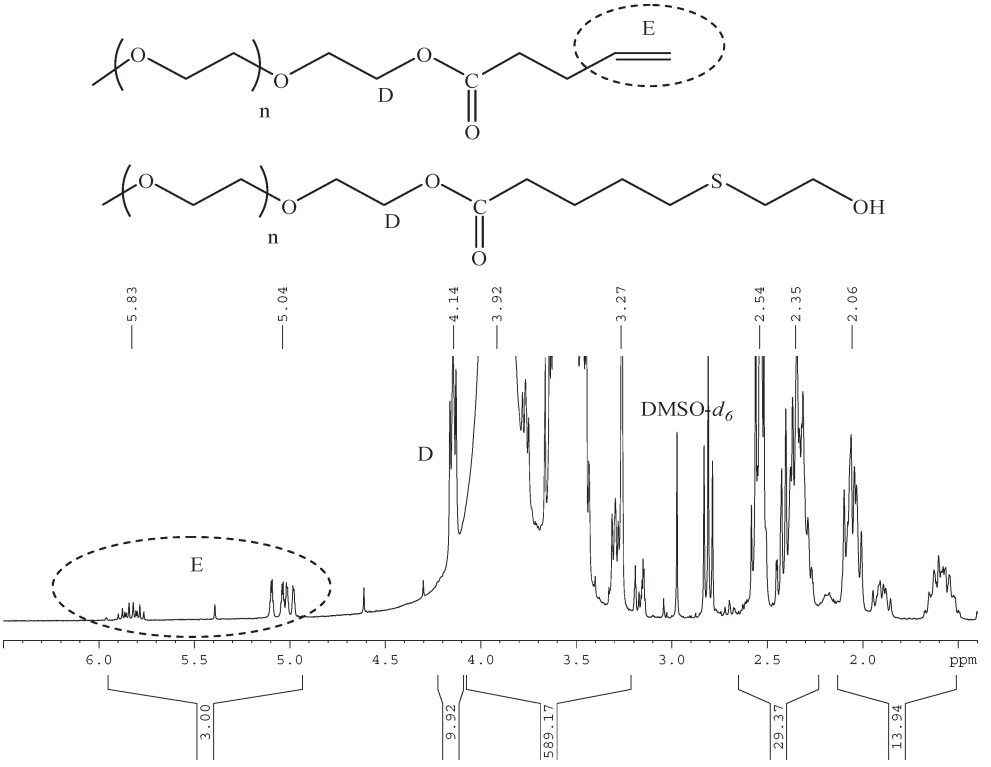


Figure 3. ^{1}H NMR spectrum of the crude reaction mixture of 2-mercaptoethanol (ME) reaction with PEG-allyl (M_n = 2000 g/mol) in DMSO-d_6. The reactions conditions: ME:PEG-allyl:DMPA:TCEP mol ratio = 100:120:20:100, 1 h at 365 nm, room temperature. The spectrum shows that ~60 mol % of PEG-allyl in the feed reacted with ME (calculated by the following equation: $[(1 - ((I_E/3)/(I_D/2)) - 0.20) \times 100]$, where I_D and I_E refer to the integration values of signals D and E, respectively, and 20 mol % corresponds to the extent of the unwanted polymerization of double bonds under the reaction conditions. Considering that the feed mole ratio of PEG-allyl:ME was 1.20, ~72 mol % of ME reacted with PEG-allyl (M_n = 2000 g/mol). Note: the peak at 5.4 ppm was due to an unknown impurity in 2-mercaptoethanol (purchased from a company and used in the reaction as received). The integration of this peak is nil; therefore, it does not affect the calculations based on peak E.

volume in the polar reaction solvent, DMSO. When a higher molecular weight PEG-acrylate (M_n = 10 000 g/mol) was used, no reaction could be observed (data not shown). This result proved that the increased steric hindrance effect lowers the grafting yield.

Thiol−Ene Reaction. The experiments for PEG grafting to PPDSM via thiol−ene reactions were performed using a PEG-allyl (M_n = 2000 g/mol). A ^{1}H NMR spectrum of PEG-allyl used for grafting via thiol−ene reactions is shown in Figure S2C (SI). PEG-allyl grafting was carried out under UV light (at 365 nm) using DMPA to induce thiol radical formation. Thiol−ene reactions can be carried out under different conditions including acid and base catalysis, nucleophilic- and radical-mediated processes, as outlined earlier. In this study, the radicals were generated by DMPA, a photoinititiator, under UV-light and then reacted with free thiols preformed concurrently from PPDSM reduction by TCEP.[28,44] First, in a control experiment, the extent of polymerization of PEG-allyl as a side reaction was tested in the absence of free thiols (PDS units:PEG-allyl:DMPA:TCEP mol ratio = 100:120:20:0, at room temperature for 1 h under UV-light at 365 nm). Approximately 20 ± 4 mol % of allyl bonds in PEG-allyl consumed under the conditions studied due to polymerization of allyl bonds of PEG-allyl and also reaction with the disulfide bonds cleaved at a small extent under UV-light (Figure S4b). Consequently, the ratio of thiol compound (either small molecular weight thiol compound or thiols created from PDS units) to PEG-allyl was set to 100:120 to compensate for the consumption of double bonds by unwanted polymerization of PEG-allyl maintaining the reagent ratios similar to those used in thiol−acrylate reactions. In a control experiment, a small molecular weight thiol (ME) and PEG-allyl were reacted (ME:PEG-allyl:DMPA:TCEP mol ratio = 100:120:20:100). The reaction yield was calculated from the disappearance of the allyl bond of PEG-allyl, as monitored via ^{1}H NMR spectroscopy. The integration value of the double bond protons was compared to that of the PEG methyl group adjacent to the ester group (CH_2−O−C(=O)−CH_2−CH_2−CH=CH_2) (Figure 3). The yield was 71.5 ± 0.5 mol % (considering the 20 mol % unwanted polymerization). The low yield (compared to base-catalyzed thiol−acrylate reactions and also other radical thiol−ene additions reported previously[31]) indicated that an excess of thiol groups is required to obtain a high yield in the radical-mediated thiol−ene reaction. However, this excess could not be used in the reaction with PPDSM to alleviate the possibility of extensive intra- and interchain disulfide bond formation. Thus, PPDSM reaction with PEG-allyl (M_n = 2000 g/mol) was tested only at 100 mol % TCEP ratio using a PEG-allyl:PDS ratio of 1.2 (PDS units:PEG-allyl:DMPA:TCEP mol ratio = 100:120:20:100). Figure 4 shows the ^{1}H NMR spectrum of the reaction mixture. The grafting yield was approximately 68 ± 2 mol % of PDS units, consistent with the yield obtained in ME and PEG-allyl reactions and also comparable with the grafting yield obtained via the thiol−acrylate reaction.

The PPDSM reaction with PEG-acrylate (M_n = 2000 g/mol) via radical addition mechanism was also tested to compare the reactivity of PEG-allyl and PEG-acrylate. The control experiment performed at a PDS units:PEG-acrylate:DMPA:TCEP mol ratio of 100:120:20:0 showed that there was no consumption of the double bonds of PEG-acrylate under the conditions without TCEP (Figure S6a, SI).

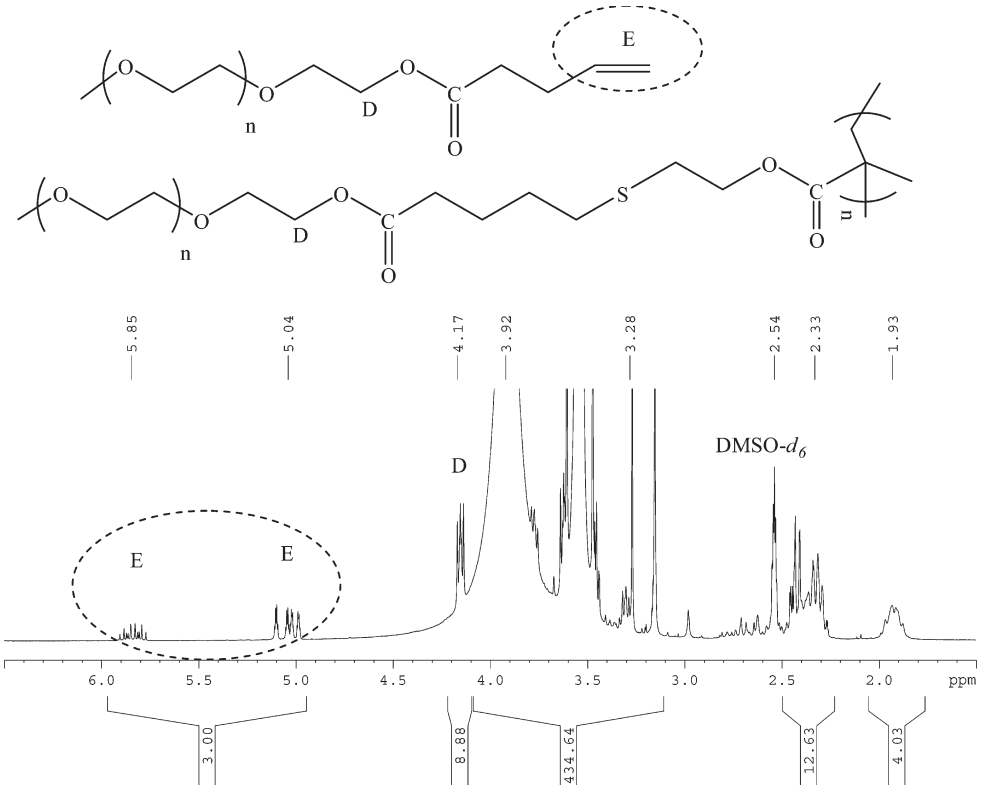


Figure 4. ^{1}H NMR spectrum of the crude mixture of PPDSM reaction with PEG-allyl (M_n = 2000 g/mol) in DMSO-d_6. The reaction conditions: PDS unit:PEG-allyl:DMPA:TCEP mol ratio = 100:120:20:100 1 h at 365 nm, room temperature. The spectrum shows that ~58 mol % of PEG-allyl in the feed reacted with PDS (calculated by the following equation: $[(1 - ((I_E/3)/(I_D/2)) - 0.20) \times 100]$, where I_D and I_E refer to the integration values of signals D and E, respectively, and 20 mol % corresponds to the extent of the unwanted polymerization of double bonds under the reaction conditions. Considering that the feed mole ratio of PEG-allyl:PDS was 1.20, ~70 mol % of PDS reacted with PEG-allyl (M_n = 2000 g/mol).

Using a molar ratio of PDS units:PEG-acrylate:DMPA:TCEP of 100:120:20:100, the grafting yield was found to be ~51 mol % of PDS units (Figure S6b, SI), which was lower compared to the yield obtained with PEG-allyl (M_n = 2000 g/mol).

Uncontrolled Reduction of PDS Groups by TCEP Leading to Cross-Linking. In a previous study, the modification of PPDSM with small molecular weight thiol compounds proved to be well-controlled via simultaneous thiol−disulfide exchange reactions.[21] Small molecular weight compounds could be grafted quantitatively to the reactive polymer via exchange of the polymer pyridyl disulfide groups with free thiol-containing compounds. The grafting strategy used in the present study differs from the previous approach in two ways: (1) within this study, the homopolymer scaffold has been grafted with a macromolecule (PEG with M_n = 454 and 2000 g/mol) rather than a small molecular weight compound, and (2) the grafting reactions (via both addition mechanisms) have occurred via two contiguous steps: the formation of free thiols (the reduction of PDS groups via a reducing agent) and then addition to double bonds. It is obvious that the grafting reaction of a macromolecule to pendant groups along a homopolymer limits the addition yield as steric hindrance increases, resulting in relatively low grafting yields compared to those observed with small molecular weight compounds.[25] On the other hand, the generation of thiols on the polymer backbone using a reducing agent may not be stoichiometric as intra- and interchain thiol−disulfide exchange reactions may occur prior to the addition of the free thiols to the ene compound. This potentially results in nonquantitative grafting and might also lead to the formation of disulfide cross-linked aggregates. This hypothesis was tested by the reduction of PDS groups with increasing amounts of the reducing agent (TCEP) in the presence of PEG-acrylate M_n = 2000 g/mol (in DMF, for 3 h, PDS units:PEG-acrylate:EA mol ratio = 100:115:3 and PDS:TCEP mol ratio was varied as 100:20, 100:40; 100:60; 100:80) and the subsequent measurement of the release of pyridine-2-thione (PT) via UV−vis spectroscopy at 374 nm. TCEP is known to be a very efficient reagent for cleaving disulfide bonds. Theoretically, 1 mol of TCEP should cleave 1 mol of PDS unit, leading to the generation of 1 mol of PT.[45] Figure 5 shows the average quantity of released PT (obtained from two independent experiments by measuring the UV absorbance of the reaction solution at 374 nm) versus the theoretical quantity of PT (calculated from the TCEP quantity used in the experiment). The experimental release of PT was consistently higher than the theoretical value targeted by the TCEP in the feed. This result shows that the generation of thiols on the homopolymer scaffold cannot be controlled by the reducing agent feed quantity. The free thiols, generated on the polymer by the reduction of PDS units with TCEP, might act instantly on other PDS units leading to a chain of inter- and intrachain thiol−disulfide exchange reactions, yielding more PT than expected (based on the TCEP used). If the free thiols generated by TCEP could react first with a PEG-acrylate rather than reacting with the other PDS groups, excess PT would not have released. However, it is clear from the data that the addition of thiols to PEG-acrylate occurs concurrently with or after the thiol−pyridyl disulfide exchange reactions. The presence of inter- and intrachain thiol−pyridyl disulfide exchange reactions potentially suggests the cross-linking of the chains via disulfide bonds. Thus, PEG (a very hydrophilic polymer) grafting to PPDSM (a hydrophobic polymer) should occur simultaneously

with inter and intrachain disulfide formation, leading to the generation of cross-linked aggregates covered with a PEG shell.

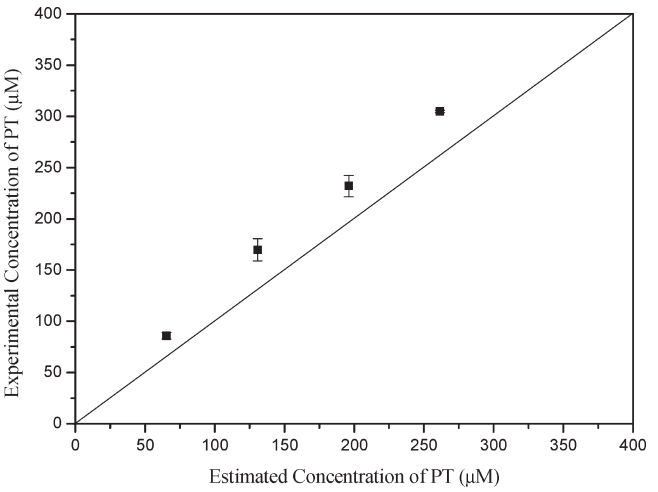


Figure 5. Reduction of PPDSM by varying amounts of TCEP (mol % of PDS groups) in the presence of PEG-acrylate (M_n = 2000 g/mol). The reaction was performed in DMF for 3 h. The concentration of pyridine-2-thione (PT) formed was determined by UV−vis spectroscopic measurement of the reaction solution (extinction coefficient = 5443 M cm^{-1} at 374 nm).[21] The measured PT concentration was plotted versus the theoretical PT concentration calculated based on the feed concentration of TCEP used to cleave PDS units. The line on the graph represents the theoretical PT release.

PEGylated Nanoparticle Formation. Dynamic light scattering (DLS) measurements of PEG-acrylate (M_n = 2000 g/mol) grafted PPDSM (74 mol % grafting yield via thiol−acrylate mechanism) in water showed the presence of particles with an average hydrodynamic diameter of 99 ± 8 nm and PDI of 0.22 ± 0.02 (Figure 6C). Atomic force microscopy (AFM) analyses of the same sample dried from a solution in water concurred with DLS showing the presence of spherical shape particles with diameters ranging from approximately ~20 to 90 nm, mostly around 30 nm (Figure 6A,B). It should be noted that the measurements obtained from AFM images reflect the size of the particles in dry state, while the DLS measurements show the hydrodynamic size of the particles in water. It would be reasonable to expect that the observed particles are covered with a PEG shell in aqueous solutions. Indeed, ^{1}H NMR analyses of the same PEG grafted PPDSM sample (PEG-acrylate M_n = 2000 g/mol; grafting yield 74 mol %) in different solvents, i.e., $CDCl_3$, D_2O, and DMSO-d_6, showed that while only PEG protons (ethylene protons of PEG: (δ = 3.62 (4H) ppm, $CH_3-(O-\mathbf{CH_2}-\mathbf{CH_2}-O-)_n-\mathbf{CH_2}CH_2-COOCH=CH_2$) are clearly visible in D_2O, PPDSM backbone protons ($Py-S-S-CH_2CH_2-COO-C-(\mathbf{CH_2})-\mathbf{CH_3}$) become gradually visible in DMSO-d_6 and $CDCl_3$, respectively. The presence of the strong PEG signals and the absence of PPDSM backbone signals in D_2O show that PPDSM backbone in water is surrounded by PEG chains (Figure 7).

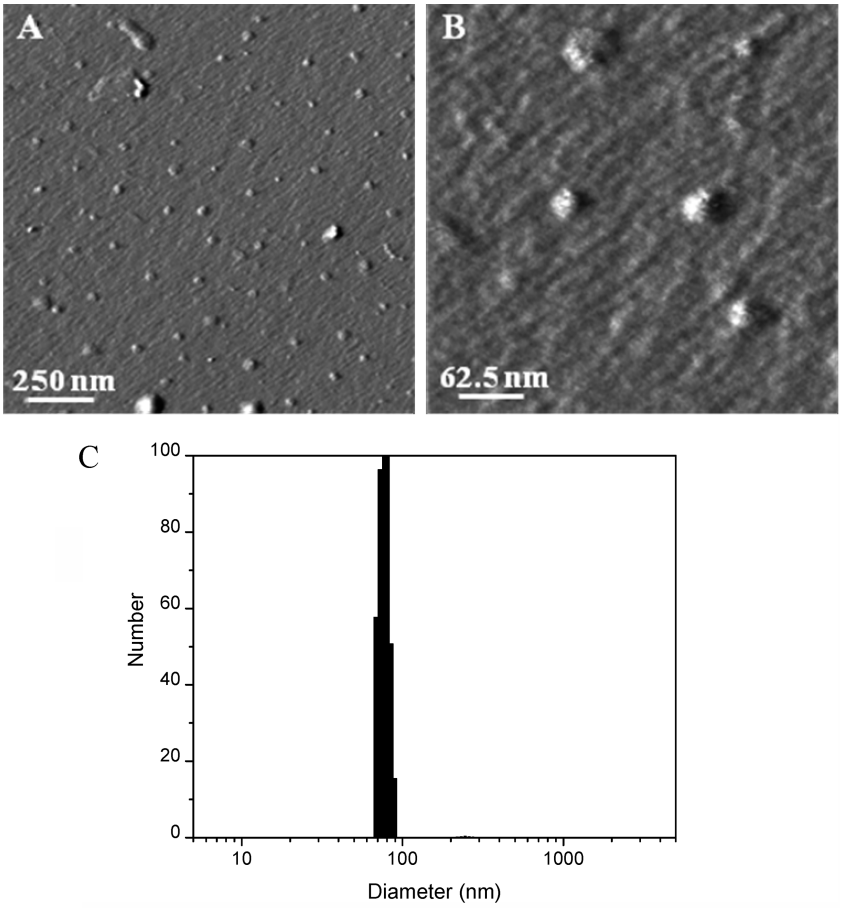


Figure 6. (A, B) AFM micrographs of PEGylated PPDSM particles (74 mol % grafting yield by thiol−acrylate addition) at different magnifications. The particle sample prepared in water was dried on a silicon substrate at atmospheric conditions. (C) Plot of DLS analysis of the same particles in water.

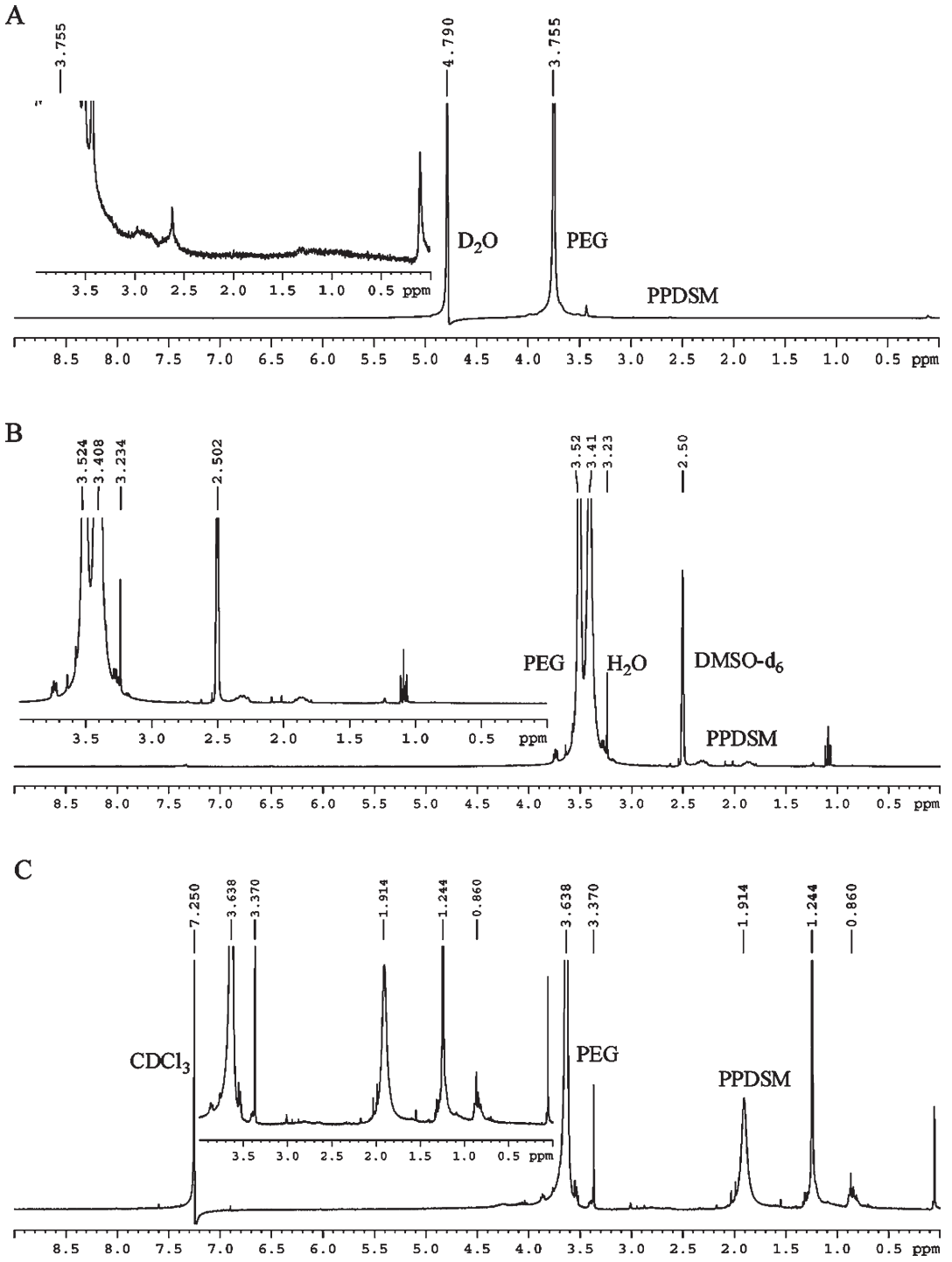


Figure 7. ^{1}H NMR spectra of PEGylated PPDSM particles (74 mol % grafting yield by thiol−acrylate addition (PEG-acrylate M_n = 2000 g/mol) in (A) D_2O, (B) DMSO-d_6, and (C) $CDCl_3$. The characteristic chemical shifts of PPDSM: the methyl/methylene protons of the polymer backbone (δ = 2.0−1.7 ppm (2H), Py−S−S−CH_2CH_2−COO−C−(**CH_2**)−CH_3, 1.1−0.7 ppm (3H), Py−S−S−CH_2CH_2−COO−C−(CH_2)−**CH_3**) and ethylene protons of PEG-acrylate: (δ = 3.62 (4H) ppm, CH_3−(O−**CH_2**−**CH_2**−O−)$_n$−**CH_2**CH_2−OOC−CH=CH_2).

Conclusions

In this study, we have exploited thiol−ene and thiol−acrylate addition reactions to convert a thiol-functional homopolymer scaffold to PEGylated nanoparticles. This synthetic approach opens a facile route to the generation of functional nanoparticles swathed with biocompatible, stealth shells for drug delivery purpose. This general approach is versatile and simple and is currently being used by our research group to develop nanoparticles for antitumor chemotherapy applications.

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Supporting Information Available: Figures S1−S6, characterization of PPDSM, NMR spectra of PEG monomethyl ether, PEG-acrylate PEG-allyl, the reaction mixture of ME

reaction with PEG-allyl, and all other supporting data for the reactions of PPDSM reaction with PEG-acrylate and PEG-allyl. This material is available free of charge via the Internet at http://pubs.acs.org.

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