Synthesis of Pyridyl Disulfide-Functionalized Nanoparticles for Conjugating Thiol-Containing Small Molecules, Peptides, and Proteins

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Previously we reported emulsion polymerization of propylene sulfide with Pluronic F127 as an emulsifier, yielding nanoparticles (NPs) in the 25 nm size range. Immunologically functional NPs were prepared by adding an antigen−Pluronic conjugate to the polymerization mixture (Reddy, S. T., et al. (2007) *Nat. Biotechnol. 25*, 1159). We sought a more flexible scheme for conjugation of antigens and other biomolecules to the NP surfaces that would allow for milder reaction conditions than achievable during the polymerization step. Here, we present the synthesis of such functionalizable NPs in the form of NPs that carry thiol-reactive groups, to which thiol-containing antigens (peptide or protein) or other biomolecules can be conjugated under mild conditions to yield immunofunctional NPs. The Pluronic-stabilized poly(propylene sulfide) (PPS) NPs with thiol-reactive pyridyl disulfide groups are prepared in two steps by (1) emulsion polymerization of propylene sulfide in the presence of a carboxylate-Pluronic and (2) reaction of the carboxylic acid groups on the NP surface with cysteamine pyridyl disulfide and a water-soluble carbodiimide reagent. We choose pyridyl disulfide groups to have a reduction-sensitive disulfide bond linking the antigen to the NP surface, allowing efficient release of antigen inside the cell in response to the reductive conditions within the endosome. The functionalizable NPs are characterized by proton NMR, dynamic light scattering (DLS), UV/vis spectroscopy, and transmission electron microscopy (TEM). Conjugation of small molecules and protein to the NP surface is presented.

INTRODUCTION

In the field of immunotherapy, one aims to either elicit or repress an immune response by directly affecting the immune system. Antigen-presenting cells (APCs[§]) patrol the body for antigens, the most powerful APCs being dendritic cells (DCs) (*1, 2*). After taking up the antigen, the DCs move to the draining lymph node to present peptides processed from the antigen and stimulate naive CD4 and CD8 T cells for induction of humoral and cell-mediated immunity (*3*). In order to induce an adaptive immune response, the DC should mature before presenting antigen to T cells. Maturation of DCs is induced by the presence of a danger signal, also referred to as an adjuvant, often selected as a ligand of Toll-like receptors (*4*).

On the basis of the above-mentioned needs, there has been growing interest in the use of biomaterials in both antigen and adjuvant delivery (*5*). Immobilizing an antigen onto or encapsulating it within the polymeric vehicle can protect the antigen from enzymatic degradation, and the carrier itself can be delivered intracellularly, enabling antigen processing and presentation to take place. Most work has focused on targeting peripheral immature DCs using polymeric poly(lactide-*co*-glycolide) (PLGA) microparticles (*6*−*12*) and polyelectrolyte microcapsules (*13*). However, these cells must travel to the draining lymph nodes to present the antigen to T cells. We previously showed that immature DCs in the lymph node can be targeted efficiently using ultrasmall nanoparticles (NPs) of about 25 nm in diameter exploiting the interstitial flow from the blood capillaries to the lymphatic capillaries and then draining lymph nodes (*14*). In this way the most potent APCs in the lymph node are targeted directly and very efficiently.

Poly(propylene sulfide) (PPS) NPs are synthesized by emulsion polymerization of propylene sulfide using Pluronic F127 an emulsifier surfactant (*15, 16*). The NPs consist of a hydrophobic rubbery cross-linked PPS core with a Pluronic-coated surface. The hydrophobic PPS core allows for encapsulation of hydrophobic drugs, should it be desired, and is stimulus-responsive toward oxidation (*16, 17*). We showed that exposure to hydrogen peroxide as a model intracellular oxidant leads to oxidation of the thioether groups leading to sulfoxide and sulfone structures, making the initial hydrophobic PPS core hydrophilic and thus soluble (*16*). This allows the incorporated drug to be released and offers a mechanism for particle clearance from the body (*17*). To show that these NPs are not only taken up by lymph-node-resident DCs but also induce adaptive immunity, we used ovalbumin (OVA) as a model antigen. OVA-NPs, prepared by incorporating an OVA−Pluronic conjugate into the NP polymerization mixture, induced both a humoral and cell-mediated immune response when injected into mice (*18*), the terminal Pluronic hydroxyl groups on the surface of the NPs activating complement to serve as an adjuvant (*18*).

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[§]Abbreviations: APC, antigen presenting cell; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; DC, dendritic cell; DLS, dynamic light scattering; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; HEPES, hydroxyethylpiperazineethanesulfonic acid; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectra; NMR, nuclear magnetic resonance; MeOH, methanol; MES, morpholineethanesulfonic acid; MWCO, molecular weight cut-off; NHS, *N*-hydroxysuccinimide; NP, nanoparticle; OVA, ovalbumin; PLGA, poly(lactide-*co*-glycolide); PO, propylene oxide; PPO, polypropylene oxide; PPS, polypropylene sulfide; sulfo-NHS, *N*-hydroxysulfosuccinimide sodium salt; TCEP·HCl, tris(2-carboxyethyl)phosphine hydrochloride; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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A limitation of the scheme described above is the need for synthesizing a Pluronic−antigen conjugate for each antigen to be bound to the NP surface. Furthermore, the antigen is linked to the NP with a bond that is not cleavable under biological conditions, which could be problematic for antigen release, especially of peptides, within DCs. We therefore sought a scheme by which to provide NPs with a reactive group that would allow straightforward conjugation of antigens and further allow release upon endocytosis by DCs. We choose thiol-reactive pyridyl disulfide groups at the surface of the NP, as antigens such as peptides and proteins can be easily engineered to contain free cysteine groups. The formed disulfide bond between the antigen and NP would allow efficient release of antigen within the cell in response to the reductive conditions within the endosome. This report describes the concept, synthesis, and characterization of thiol-reactive, reductively sensitive NPs and the conjugation with low molecular weight compounds and proteins.

EXPERIMENTAL PROCEDURES

Materials and Methods. Pluronic F127, hydroxyethylpiperazineethanesulfonic acid (HEPES) (Sigma), streptavidin-Alexa488 (Invitrogen), (+)-D-biotin (Applichem), aldrithiol, fluorescamine, trifluoracetic acid (TFA), triisopropylsilane (TIS), phenol, 0.5 M $NaOCH_3$ in methanol, propylene sulfide, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N*-hydroxysuccinimide (NHS), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), iodoacetamide, potassium hydroxide, CaH_2 (Aldrich), dithiothreitol (DTT) (Biorad), Sephadex G50, Sepharose 6B, Sepharose CL-6B (Sigma), Ellman reagent (Pierce), cysteamine hydrochloride, potassium carbonate, mercaptopropionic acid methyl ester, triphenylphosphine, acetic acid, methanesulfonyl chloride, sodium hydroxide, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), triphenylmethanol, $BF_3 \cdot Et_2O$ (Fluka), and morpholineethanesulfonic acid (MES) (Calbiochem) were used as received. All chemicals relating to peptide synthesis were from Novabiochem. Molecular sieves (3 Å, Fluka) were heated at 180 °C under reduced pressure for at least 16 h. Diethyl ether, methanol, and toluene were used as received. Dimethylformamide (DMF) was distilled over CaH_2 under reduced pressure at 40 °C and kept over molecular sieves. Dichloromethane was dried over molecular sieves. Water was distilled twice or freed from salt using the Milli-Q water system. All reactions were done under argon (Messer) atmosphere.

^1H NMR. Spectra were measured on a Bruker 400 Ultrashield spectrometer. NMR was performed in deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD), or deuterated dimethyl sulfoxide (DMSO-d_6). Peaks are referenced against residual solvent at 7.26 ppm ($CDCl_3$), 3.35 ppm (CH_3OD), or 2.50 ppm (DMSO-d_6). For each sample 32 scans were collected and the D1 was set at 10 s.

UV/Visible Spectroscopy. Spectra were obtained on a Shimadzu UV mini 1240 UV/visible spectrophotometer using 1.0 cm quartz cuvettes or a Tecan Safire^2 well plate reader using polystyrene well plates.

Dynamic Light Scattering. Data were obtained on a Malvern Nanoseries Zetasizer instrument with 632 nm laser using PMMA cuvettes by diluting NP solutions with PBS (10 mM, pH 7.4) to a final NP concentration of 2 mg/mL. Sizes are given in nm and are obtained as the *Z*-average by fitting the correlation function using the cumulant method.

High Resolution Mass Spectrometry. Spectra were collected by the Institute of Chemical Sciences and Engineering (ISIC) at the Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland. ESI-TOF-MS data were acquired on a Q-Tof Ultima mass spectrometer (Waters) fitted with a LockSpray dual electrospay ion source and operating in the positive ionization mode. The samples were diluted in methanol acidified with 0.1% HCOOH and introduced into the mass spectrometer by infusion at a flow rate of 20 mL/min. External calibration was carried out with a solution of phosphoric acid at 0.01%. Data were processed using the MassLynx 4.1 software.

Transmission Electron Microscopy. Carbon coated 400 mesh copper grids (Agar Scientific, Stansted, U.K.) were prepared under glow discharge, and 5 μL of NP solution was placed onto the grid. After 60 s, the grid was washed with H_2O and then dried by blotting the side of the grid with filter paper. To stain, 5 μL of 2% uranyl acetate solution was added to the grid. After 30 s, the stain was blotted off the same as above and was allowed to dry for 5 min at room temperature before analysis. Microscope images were acquired on a Philips CM-12 TEM (Koninklijke Philips Electronics, Eindhoven, The Netherlands). Images were captured using a Gatan MSC CCD camera (Gatan Inc., Grandchamp, France) and processed using Adobe Photoshop software (Adobe Systems, San Jose, CA). TEM images were analyzed with Scion image analysis software, and size results are given as the mean diameter ± SD and number of particles measured in parentheses.

Preparative HPLC. Purification by HPLC was done by reverse phase HPLC chromatography using a C18 column. Separation and collection were performed by UV and mass directed software (Waters, Baden-Daettwil, Switzerland).

SDS−PAGE. The 10% gels were run at 300 V for 2 h. Protein bands were visualized by UV.

Ellman Assay (19). Ellman solution was prepared by dissolving 4.0 mg of Ellman reagent in 1.0 mL of Ellman buffer (1 mM EDTA, 100 mM phosphate, pH 8.0). Thiol concentrations were measured by reacting 20 μL of Ellman solution with 20 μL of sample. After 15 min the mixture was diluted by the addition of 960 μL of Ellman buffer and the absorbance at 412 nm read. Thiol concentrations were calculated from a standard curve using thiomaleic acid. In the range 1−4 mM measurements were done in a well plate using 200 μL of sample. For thiol concentrations in the range 50−1000 μM, measurements were done using a quartz cuvette.

Fluorescamine Assay (20). Fractions from the Sepharose 6B and Sepharose CL-6B column (50 μL) were mixed with 100 μL of 100 mM HEPES, pH 8, and 20 μL of a solution of fluorescamine (5 mg/mL) in acetone. The sample was reacted at room temperature for 15 min and the fluorescence (λ_{ex} = 390 nm, λ_{em} = 475 nm) read using black polystyrene Nunc plates.

Pyridyl Disulfide Loading NPs. Pyridyl-NP solutions were mixed 1:1 by volume with a 5 mM TCEP·HCl solution in 100 mM HEPES, pH 8, and reacted for 15 min. The absorbance at 340 nm was read, and after correction for the absorbance of pyridyl-NP and TCEP·HCl the concentration of pyridyl disulfide calculated from a standard curve of pyridine thione (**8**) in HEPES, pH 8. If the concentration was too high, NP solutions were diluted with H_2O.

Compound Synthesis. *Pluronic Bromide (**1**).* **1** was prepared as reported before (*21*).

*Pluronic Mesylate (**2**).* An amount of 23.6 g (1.86 mmol) of Pluronic F127 was dissolved in 500 mL of toluene, and 100 mL of toluene was removed by azeotropic distillation using a Dean−Stark trap. The solution was cooled to room temperature, and 2.6 mL (18.6 mmol, 10 equiv) of triethylamine and 1.4 mL (18.6 mmol) of methanesulfonyl chloride was added. After 18 h the yellow suspension was filtered and the clear yellow filtrate concentrated under reduced pressure. The residue was dissolved in 100 mL of CH_2Cl_2, filtered over a SiO_2 bed (1.0 cm (diameter) × 5.0 cm (height)), and the SiO_2 was washed with CH_2Cl_2 (3 × 50 mL). The filtrate was concentrated to a

volume of about 50 mL and precipitated in 1000 mL of Et_2O. The white precipitate was filtered off, washed with 100 mL of Et_2O, and dried under reduced pressure to yield 21.0 g (89%) of white powder. The degree of functionalization was 100% according to ^{1}H NMR. ^{1}H NMR ($CDCl_3$): $\delta = 4.35-4.38$ (m, CH_2-O-SO_2-), 3.60−3.67 (m, CH_2, PEG), 3.47−3.58 (m, CH_2, PPO), 3.34−3.42 (m, CH, PPO), 3.07 (*s*, -SO_2-C$\underline{H}_3$), 1.11−1.15 (CH_3, PPO).

*Pluronic Thioether Propionic Acid Methyl Ester (**3**).* An amount of 21.0 g (**2**) was dissolved in 100 mL of DMF, and to the clear solution was added 2.29 g (16.5 mmol) of potassium carbonate followed by 2 mL (16.5 mmol) of mercaptopropionic acid methyl ester. The mixture was stirred for 20 h and concentrated under reduced pressure. The residue was dissolved in 100 mL of CH_2Cl_2 and mixed with 11 g of activated carbon. The mixture was filtered over a filter cell and the residue washed with CH_2Cl_2 (3 × 100 mL). The clear colorless filtrate was reduced to about 50 mL by evaporation and precipitated in 1000 mL of Et_2O. The white solid was filtered, washed with 100 mL of Et_2O, and dried under reduced pressure to yield 18.2 g (87%). The degree of functionalization was 92% according to ^{1}H NMR. ^{1}H NMR ($CDCl_3$): $\delta = 3.70$ (s, OCH_3), 3.59−3.63 (m, CH_2, PEG), 3.46−3.58 (m, CH_2, PPO), 3.36−3.44 (m, CH, PPO), 2.83 (*t*, $J = 7.6$ Hz, -O-CH_2-C$\underline{H}_2$-S-), 2.73 (*t*, $J = 7.2$ Hz, −S-C$\underline{H}_2$-CH_2-CO-), 2.63 (t, $J = 7.2$ Hz, -CH_2-CO-), 1.12−1.16 (CH_3, PPO).

*Pluronic Thioether Propionic Acid Sodium Salt (**4**).* An amount of 18.0 g (**3**) (2.83 mmol) was dissolved in 200 mL of Milli-Q water, and to the clear solution added a solution of 0.57 g (14.1 mmol) of sodium hydroxide in 5 mL of Milli-Q water. After 21 h the solution was transferred into a regenerated cellulose dialysis tube, molecular weight cut-off (MWCO) of 3.5 kDa, and dialyzed against 5 L of Milli-Q for 29 h with replacement of the water three times. The solution was lyophilized to yield 12.2 g (68%) of white solid. The degree of functionalization according to ^{1}H NMR was 95%. NMR ($CDCl_3$): $\delta = 3.59-3.63$ (m, CH_2, PEG), 3.46−3.58 (m, CH_2, PPO), 3.35−3.44 (m, CH, PPO), 2.83 (t, $J = 6.4$ Hz, -O-CH_2-C$\underline{H}_2$-S-), 2.73 (t, $J = 6.8$ Hz, -S-C$\underline{H}_2$-CH_2-CO-), 2.50, (t, $J = 6.8$ Hz, -CH_2-CO-), 1.13−1.16 (CH_3, PPO).

*Pyridyl Disulfide Cysteamine HCl Salt (**5**).* An amount of 0.57 g (0.5 mmol) of cysteamine·HCl was added to a solution of 3.3 g (15.0 mmol) of aldrithiol in 10 mL of methanol. The yellow solution was stirred overnight and the product obtained by precipitation in 500 mL of Et_2O. After filtration, the residue was dissolved in 15 mL of MeOH and again precipitated in 500 mL of Et_2O. The white solid was dried under reduced pressure to yield 0.94 g (84%). ^{1}H NMR (DMSO-d_6): $\delta = 8.51-8.50$ (m, 1H), 8.30 (bs, 3H), 7.86−7.82 (m, 1H), 7.75 (d, 1H), 7.31−7.28 (m, 1H), 3.13−3.05 (m, 4H). HRMS $[M + H]^+$ calculated for $C_7H_{10}N_2S_2$ 187.0364, found 187.0358.

*Pluronic Pyridyl Disulfide (**6**).* Amounts of 0.25 g (0.02 mmol) of **4** and 22.8 mg (0.2 mmol) of NHS were dissolved in 5 mL of CH_2Cl_2. To the clear solution were added 38 mg (0.2 mmol) of EDC, 44 mg (0.2 mmol) of **5**, and 38 μL (0.2 mmol) of triethylamine, and the turbid mixture was stirred for 18 h at room temperature. The yellow solution was concentrated in a flow of N_2 and the residue dissolved in 5 mL of H_2O. The milky solution was transferred to a dialysis bag (MWCO = 3.5 kDa) and dialyzed against 2 L of H_2O for 1 day. The polymer was recovered by lyophilization to yield 0.19 g (76%). The degree of functionalization according to ^{1}H NMR was 95%. ^{1}H NMR ($CDCl_3$): $\delta = 8.51$ (m, CH_{aromat}), 7.64 (m, CH_{aromat}), 7.55 (m, CH_{aromat}), 7.33 (bs, NH), 7.13 (m, CH_{aromat}), 3.69−3.59 (m, CH_2, PEG), 3.58−3.44 (m, CH_2, PPO), 3.42−3.33 (m, CH, PPO), 2.94 (t, CH_2, $J = 5.6$ and 6.0 Hz), 2.88 (t, CH_2, $J = 7.2$ Hz), 2.74 (t, CH_2, $J = 6.8$ and 6.4 Hz), 2.51 (t, CH_2, $J = 7.2$ and 7.6 Hz), 1.14 (m, CH_3, PPO).

Table 1. Dynamic Light Scattering Results for the Different NP Formulations[a]

	carboxylate-NP		pyridyl disulfide-NP	
wt % COOH	*Z*-avg[b] (SD)	PDI[c] (SD)	*Z*-Avg[b] (SD)	PDI[c] (SD)
0	31.8 (0.80)	0.260 (0.003)		
10	33.0 (0.31)	0.142 (0.006)	35.7 (0.13)	0.207 (0.005)
25	31.0 (0.24)	0.168 (0.013)	33.7 (0.71)	0.175 (0.003)
50	32.6 (0.08)	0.147 (0.004)	42.5 (0.30)	0.225 (0.009)
75	32.9 (0.18)	0.145 (0.006)	42.0 (0.15)	0.219 (0.002)
100	31.3 (0.22)	0.113 (0.001)	42.4 (0.17)	0.209 (0.004)

[a] Values are the average of three measurements of the same sample. [b] Size in nm as measured by the *Z*-average obtained by fitting the correlation function using the cumulant method. [c] Polydispersity index (PDI) calculated from fitting the correlation function using the cumulant method.

*Thioacetic Acid S-[3-[3-(3-Acetylsulfanylpropoxy)-2,2-bis-(3-acetylsulfanylpropoxymethyl)propoxy]propyl] Ester (**7**).* The four-arm initiator was synthesized as reported (*16*).

NP Synthesis. This was adapted from that reported (*16*). An amount of 10 mL of Milli-Q H_2O was freed from oxygen by three evacuation/purge (Ar) cycles, and under Ar flow the hydroxyl- and carboxylate Pluronic (500 mg total) were added. After all the Pluronic was dissolved the resulting solution was again evacuated and purged with Ar three times, and under Ar flow 400 μL (5.0 mmol) of propylene sulfide was added. The emulsion was stirred for 30 min after which 24.3 mg (0.04 mmol) of the four-arm initiator **7**, previously reacted for 15 min with 322 μL of 0.5 M $NaOCH_3$ (0.16 mmol) solution in MeOH, was added under Ar. The mixture was stirred for another 15 min, and then an amount of 60 μL of DBU (0.4 mmol) was added. The mixture was stirred for 24 h and exposed to air for 2 h to cross-link, and the remaining thiolates were quenched by the addition of 47.5 mg (0.26 mmol) of iodoacetamide and stirred for 2 h. Ellman assay of the NP solution confirmed the absence of thiolate anions, and the mixture was dialyzed (MWCO = 100 kDa) against 5 L of Milli-Q water for at least 2 days with regular replacement of the water. After filtration (0.22 μm filter) the size of the NPs was measured by dynamic light scattering and a small volume was lyophilized to obtain the NP concentration in mg/mL. NMR of the solid was used to calculate the weight % of Pluronic in the NPs.

*Pyridinethione (**8**).* The ether fractions of **5** were combined and concentrated under reduced pressure, and the residue was dissolved in 10 mL of MeOH and reacted with 1.74 g (15.0 mmol) of cysteamine·HCl overnight. The turbid mixture was diluted with 100 mL of EtOAc and washed with 100 mL of H_2O. After the organic phase was dried over Na_2SO_4 the mixture was filtered and the clear solution concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography, $R_f = 0.46$ (EtOAc). ^{1}H NMR ($CDCl_3$): $\delta = 7.61-7.56$ (m, 1H), 7.43−7.39 (m, 1H), 6.81−6.78 (m, 1H), 1.79 (bs, 1H). HRMS $[M + H]^+$ calculated for C_5H_5NS 112.0221, found 112.0297.

Synthesis of Pyridyl Disulfide-NPs, General Procedure. To the carboxylate-NP solution is added solid MES to a final concentration of 100 mM and the pH adjusted to 4.0−4.5 with a pH meter. To the clear solution are added pyridyl disulfide cysteamine·HCl, sulfo-NHS, and EDC all at an excess of 20 equiv relative to the amount of Pluronic as calculated from NMR. After reaction for 2−24 h the solution is dialyzed for at least 2 days to remove unreacted reagents using MWCO = 3.5−10 kDa. The pyridyl disulfide-NPs in Table 1 and Figures 2−4 were made by reacting for 24 h and then purified using MWCO = 3.5 kDa dialysis tubing. Below follows the detailed reaction for functionalization of 100 wt % carboxylate-NPs. To

3.0 mL of 100 wt % carboxylate-NP solution (50.4 mg/mL), corresponding to 28.7 mg/mL of Pluronic according to NMR, was added 60 mg of MES (final concentration 102 mM), and the pH was adjusted to 4.4. To the NP solution containing 86.1 mg of carboxylate-Pluronic (0.014 mmol of carboxylate groups) were added 30.1 mg (0.14 mmol, 10 equiv) pyridyl disulfide cysteamine·HCl salt, 30.1 mg of sulfo-NHS (0.14 mmol, 10 equiv), and 33.1 mg (0.17 mmol, 12 equiv) of EDC. After 22 h the NP solutions were dialyzed against 5 L of Milli-Q water (MWCO = 3.5 kDa) for 2 days. After filtration through 0.22 μm filter, the size was measured by dynamic light scattering. The concentration of the pyridyl disulfide groups was determined by measuring the absorbance at 340 nm after reducing the NPs with TCEP·HCl. Lyophilization of a small sample yields the concentration of the NP solution.

*Trityl Cysteamine (**9**).* An amount of 11.4 g (100 mmol) of cysteamine·HCl and 27.3 g (105 mmol) of triphenylmethanol was added to 100 mL of DMF, and the mixture was heated at 60 °C for 15 min to obtain a clear-yellow solution. The solution was cooled, 14 mL of BF_3·Et_2O (110 mmol) was added, and the mixture was heated at 80 °C. After 14 h the mixture was concentrated under reduced pressure and the oil was dissolved in 500 mL of EtOAc. Upon standing a white solid precipitated that was filtered off. The remaining clear EtOAc solution was washed with saturated NaCl (aq) solution, causing more solid to precipitate that was filtered off. Both solids were combined and dried under reduced pressure, yielding 33.1 g (93 mmol, 93%) of tritylcysteamine·HCl salt. The free base was prepared by treating 2.9 g (8.1 mmol) of the HCl salt with 150 mL of saturated KOH (aq) solution and 500 mL of Et_2O. The clear layers were separated, and the organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. This yielded after drying under reduced pressure 2.0 g (8.3 mmol, 78%) of the free salt. NMR ($CDCl_3$): δ = 7.44 (m, 6H, CH_{aromat}), 7.27 (m, 9H, CH_{aromat}), 2.60 (t, 2H, CH_2−N, J = 6.4 and 6.6 Hz), 2.34 (t, 2H, CH_2-S, J = 6.4 and 6.6 Hz), 1.48 (br, 2H, NH_2). HRMS $[M + H]^+$ calculated for $C_{21}H_{21}NS$ 320.1473, found 320.1466.

*Biotin Cysteamine (**10**).* Amounts of 0.49 g (2.0 mmol) of D-(+)-biotin, 0.47 g (2.0 mmol) of EDC, and 0.96 g (5.0 mmol) of *N*-hydroxysuccinimide were suspended in 10 mL of DMF. After the addition of 0.58 g (5.0 mmol) of **9** and 278 μL (2.0 mmol) of triethylamine the mixture was stirred for 2 days at room temperature. The mixture was concentrated under reduced pressure and taken up in 50 mL of EtOAc and washed with H_2O (3 × 50 mL). After drying over Na_2SO_4 and solvent removal the residue was dissolved in a mixture of 10 mL of CF_3COOH and 10 mL of CH_2Cl_2, and 431 μL (2.1 mmol) triisopropylsilane was added. After 4 h the volatiles were removed in a flow of N_2 and the residue was dissolved in 25 mL of EtOAc and extracted with H_2O (3 × 10 mL). The aqueous extracts were combined and lyophilized. The compound was purified by HPLC to yield 0.091 g of white solid (15% overall yield). ^{1}H NMR (CD_3OD): δ = 4.53 (m, 1H), 4.34 (m, 1H), 3.37 (t, 2H, J = 6.8 Hz), 3.25 (m, 1H), 2.97 (dd, 1H, J = 4.8 and 12.81 Hz), 2.74 (d, 1H, J = 12.8 Hz), 2.64 (t, 2H, J = 7.2 Hz), 2.25 (t, 2H, J = 7.2 Hz), 1.82−1.58 (m, 4H), 1.54−1.42 (m, 2H). HRMS $[M + H]^+$ calculated for $C_{12}H_{21}N_3O_2S_2$ 304.1154, found 304.1157.

Biotin-NPs. Pyridyl disulfide-NPs (500 μL, 35.2 mg/mL, 75% NP) with a pyridyl disulfide loading of 1.9 mM were diluted with 500 μL of HEPES solution (200 mM, pH 8.0) and added to 1.1 mg (3.6 μmol, 1.8 equiv) of biotin cysteamine. The reaction was followed by measuring the absorbance at 340 nm in a quartz cuvette. After 1 h the absorbance was stable and the NPs were purified by dialysis (MWCO = 10 kDa) against 5 L of H_2O for 24 h with regular replacement of water. The presence of biotin on the NPs was confirmed by the HABA/avidin displacement assay (*22*). The number of biotin molecules per NP was estimated to be approximately 800 (see Supporting Information).

*Ac-SDKDSLKCG-OH Peptide (**11**).* Fmoc-Gly (0.65 mmol, 194.0 mg, 3 equiv) was coupled to NovaPEG Wang resin (250 mg, loading 0.87 mmol/g) with 134.6 mg (0.65 mmol, 3 equiv) of DCC and 13.2 mg (0.11 mmol, 0.5 equiv) of DMAP in 10 mL of DMF. After the resin was washed with DMF (5 × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (10 mL) and after washing with DMF (5 × 10 mL) used to couple the other amino acids using standard Fmoc-amino acid/HBTU/HOBt/DIPEA (0.5 M/0.5 M/0.5 M/1.0 M, 5 equiv solutions in DMF) double coupling Fmoc chemistry on a Chemspeed peptide synthesizer. After Fmoc removal of the last amino acid serine the N-terminus was acetylated by reaction with acetic acid/HBTU/HOBt/DIPEA. The peptide was cleaved from the resin using 4 mL of TFA/TIS/phenol/H_2O, 35:2:2:1, for 4 h and precipitated in diethyl ether to yield the crude peptide that was purified by reverse phase HPLC to yield 71 mg (0.064 mmol, 29%) of white solid. HRMS $[M + H]^+$ calculated for $C_{43}H_{12}N_2O_{20}S$ 1109.4785, found 1109.4803.

Peptide-NPs. A solution of the peptide was prepared at a thiol concentration of 2.1 mM, and 100 μL of this solution was diluted with 100 μL of 100 mM acetate buffer, pH 4, and added to 200 μL of pyridyl-NP solution (58.8 mg/mL, 25% NP) with a pyridyl disulfide loading of 0.93 mM. The excess of thiol to pyridyl disulfide was 10%. The reaction was followed by measuring the absorbance at 340 nm in a well plate reader. After 2 h the absorbance was stable and the peptide-NPs were purified by gel filtration over Sepharose 6B beads eluting in water (200 μL sample and 10 mL bed volume). Presence of the peptide on the NPs was measured with fluorescamine. The number of Ac-SDKDSLKCG-OH peptide molecules per NP was estimated to be approximately 160 (see Supporting Information).

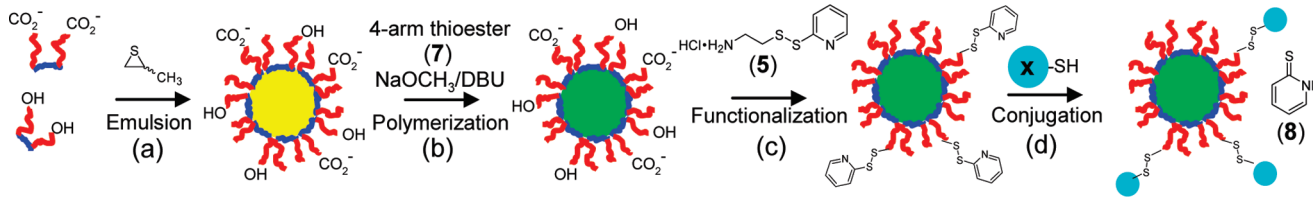
Reduction of Peptide-NPs. An amount of 200 μL of purified peptide-NPs was mixed with 10 μL of a solution of 57.2 mg/mL TCEP·HCl, resulting in a final concentration of 9.5 mM reducing agent. The mixture was incubated for 2 h, and the NPs were purified by gel filtration over Sepharose 6B beads eluting in water (200 μL sample and 10 mL bed volume). As a control, 200 μL of purified peptide-NPs mixed with 10 μL of H_2O was purified as well.

OVA-Functionalized NPs. OVA (12.5 mg/mL) in 10 mM PBS was reduced with DTT at a final concentration of 32 mM for 4 h. Excess reducing agent was removed by gel filtration over Sephadex G50 eluting with H_2O. The fractions were analyzed by Ellman assay prior to lyophilization to quantify the amount of OVA; it was assumed that all cysteines were present in the thiol form. Pyridyl disulfide-NPs with a pyridyl disulfide loading of 547 μM (41 mg/mL, 25% NP) were added to 750 μL of lyophilized 100 mM HEPES, pH 8.0, solution, and the clear solution was added to 7.85 mg of lyophilized OVA resulting in a final OVA concentration of 10.4 mg/mL (236 μM). The reaction mixture was incubated overnight at 4 °C and purified by gel filtration over Sepharose CL-6B beads eluting in water (180 μL sample and 12 mL bed volume). As a control, nonreduced OVA was reacted in the same way. Presence of the OVA protein was measured with fluorescamine. The number of OVA protein molecules per NP was estimated to be approximately 75 (see Supporting Information).

RESULTS

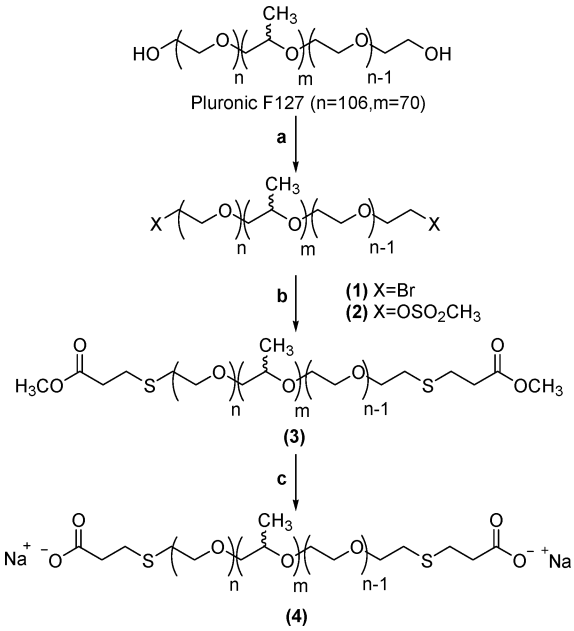
In Scheme 1, the design concept of the functionalizable NPs is shown. When a carboxylate-Pluronic is mixed with the hydroxyl-Pluronic into the propylene sulfide emulsion, NPs can be prepared with surface carboxylate groups. These carboxylate

Scheme 1. Synthesis of Funtionalizable Nanoparticles[a]



[a] (a) Hydroxyl-Pluronic and carboxylate-Pluronic are used to make an emulsion with propylene sulfide. (b) To the emulsion is added four-arm thiol initiator, and polymerization is started by addition of base. After polymerization, particles are exposed to air to cross-link, and the remaining thiolates are end-capped with iodoacetamide. (c) The carboxylate-nanoparticles are reacted with pyridyl disulfide cysteamine (**5**) to yield thiol-reactive functionalizable nanoparticles. (d) Conjugation of thiol-containing biotin (**10**), AcSDKDSLKCGOH peptide (**11**), and OVA protein gives biological-functional nanoparticles.

Scheme 2. Synthesis of Carboxyl-Functionalized Pluronic[a]



[a] (a) $SOBr_2$, toluene, reflux 4 h (**1**) or CH_3SO_2Cl, Et_3N, toluene, overnight (**2**); (b) thiopropionic acid methyl ester, K_2CO_3, DMF, overnight; (c) NaOH (aq), overnight.

groups on the NP surface can then be converted into pyridyl disulfide groups. The thiol-reactive pyridyl disulfide group allows for conjugation with thiol-containing small molecules, peptides, and proteins. The first step is synthesis of carboxylate group-carrying NPs. To accomplish this, a Pluronic-carboxylate was synthesized as shown in Scheme 2.

As the first step of Pluronic carboxylation, both hydroxyl groups were converted into either the bromide by reaction with thionyl bromide or the mesylate with methanesulfonyl chloride in toluene. The degree of functionalization was 100% for both the bromide and mesylate according to NMR, as no OH groups were visible in deuterated DMSO. Harris et al. showed that the OH group of PEG occurs as a triplet at $\delta = 4.58$ ppm in this aprotic solvent and can be used to determine the degree of functionalization reliably (*23*). Nucleophilic substitution of the bromide or mesylate with mercaptomethyl propionate in the presence of potassium carbonate in DMF afforded the corresponding methyl ester in high yield. Hydrolysis of the ester in aqueous NaOH yielded the carboxylate-Pluronic, as was shown by the disappearance of the CH_3 group at 3.7 ppm. The presence of the carboxylate groups was furthermore confirmed by reaction with pyridyl disulfide cysteamine·HCl in the presence of EDC and NHS (Scheme 3) to yield the corresponding pyridyl disulfide-Pluronic. In addition, the degree of functionalization was calculated by reducing the pyridyl disulfide groups with TCEP·HCl in aqueous HEPES, pH 8, and reading the absorbance at 340 due to pyridinethione release. The degree of functionalization was 100% on a mass basis.

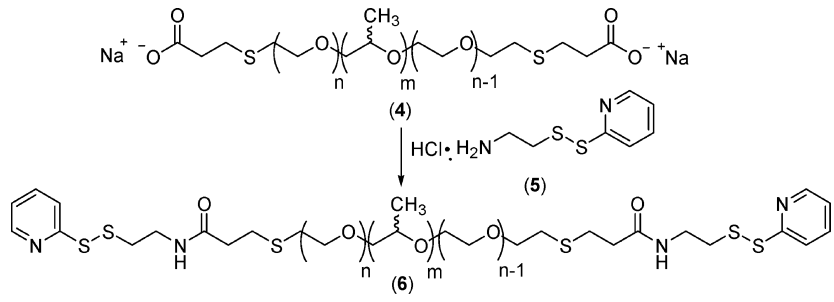
The synthesized carboxylate-Pluronic was used for NP synthesis. To gain information on how it would affect NP synthesis, the weight ratio of carboxylate-Pluronic to hydroxyl-Pluronic in the polymerization mixture was varied between 0% and 100%. (For some applications, retention of surface hydroxyl groups on the NPs is important (*18*).) DLS and TEM show that the addition of carboxylate-Pluronic does not significantly affect the size of NPs compared to the NPs with hydroxyl-Pluronic alone, as can be seen from Table 1 and Figure 1, panels A and B. However, the polydispersity index (PDI) was lower than for NPs with hydroxyl-Pluronic alone, which is reflected by the absence of the larger particles in 100% carboxylate-NPs (Figure 1B) and their presence in the hydroxyl-NPs (Figure 1A).

From NMR, the degree of polymerization of propylene sulfide can be calculated by comparing the integral ratio after normalizing of the propylene sulfide CH_3 group at 1.3 ppm and that of the four-arm initiator methylene group -CH_2- at 1.8 ppm. Figure 2 shows the degree of polymerization as a function of weight percentage carboxylate-Pluronic present in the polymerization mixture. The average degree of polymerization for the different carboxylate-NPs is 63 ± 3 propylene sulfide monomer units, which corresponds to 16 ± 1 units per initiator arm in average. These values are quite similar to those (58 and 14 units, respectively) calculated for hydroxyl-NPs. NMR also allows calculation of the weight contributions of Pluronic, PPS, and initiator to the total NP mass (see Supporting Information). From Figure 3 it can be seen that the different carboxylate-NPs all have a similar composition. The average values are $57 \pm 2.1\%$ for Pluronic, $39 \pm 2.0\%$ for PPS, and $3.6 \pm 0.2\%$ for the initiator. These values agree well with those found for hydroxyl-Pluronic alone NPs (55%, 41%, and 4.1%, respectively).

Having established that NPs can be made using the carboxylate-Pluronic, the next step was reacting them with pyridyl disulfide cysteamine·HCl, obtained in a single step from cysteamine·HCl and aldrithiol in methanol, using standard EDC/sulfo-NHS coupling chemistry at pH 4.0−4.5. The amount of Pluronic was calculated from NMR data of lyophilized carboxylate-NPs. Since it was not clear what the amount of carboxylate-Pluronic in the 10−75% carboxylate-NPs was, calculations of coupling reagents were based on the total amount Pluronic in the NP solutions (as measured by NMR), which was similar for all carboxylate-NPs solutions. The number of equivalents of EDC (24 equiv), sulfo-NHS (20 equiv), and pyridyl disulfide cysteamine·HCl (20 equiv) relative to Pluronic was fixed, and the reactions were carried out at the same time.

After functionalization, the NPs were dialyzed and characterized by DLS (Table 1) and TEM (Figure 1C). The PDI slightly increased, and TEM supports the DLS data, as larger particles are present as well. NMR of the different NPs revealed the presence of the pyridyl disulfide group by the four characteristic signals of the pyridyl disulfide group as observed for the pyridyl

Scheme 3. Synthesis of Pyridyl Disulfide-Functionalized Pluronic[a]



[a] EDC, NHS, CH_2Cl_2, overnight.

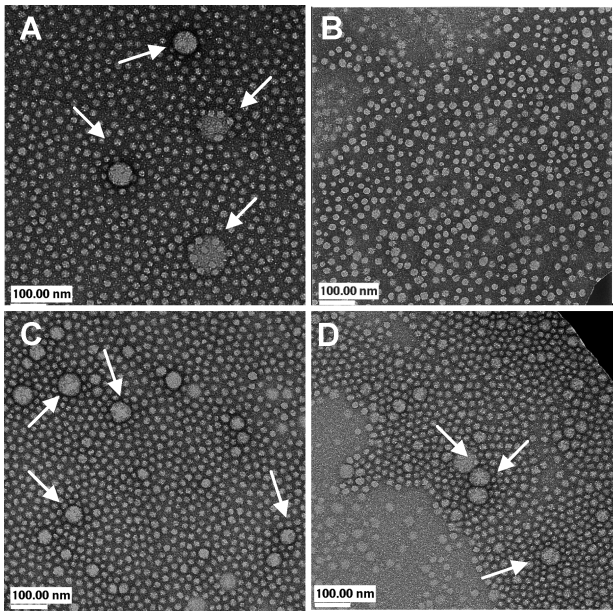


Figure 1. TEM images of aqueous nanoparticle (NP) suspensions. Size ± SD is given in nm, and number of particles measured is indicated in parentheses: (A) hydroxyl-NPs, 17.4 ± 4.5 (691); (B) 100% carboxylate-NPs, 16.0 ± 3.6 (570); (C) 50% pyridyl disulfide-NPs, 16.2 ± 4.5 (986); (D) 75% biotin-NPs, 16.7 ± 5.3 (849). Some larger particles as mentioned in the text are indicated by white arrows.

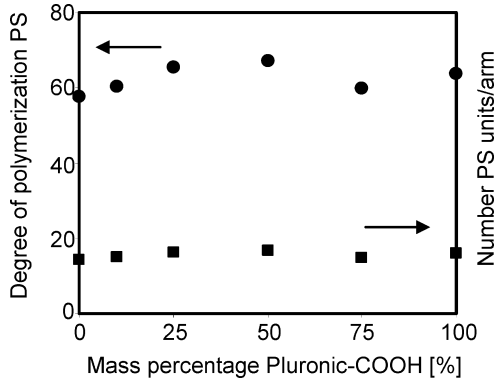


Figure 2. Degree of polymerization of propylene sulfide (circles) and number of propylene sulfide units per initiator arm (squares) as a function of weight percentage carboxylate-Pluronic in the polymerization mixture.

disulfide-Pluronic (**6**). More importantly, the presence of a broad signal at 7.3 ppm assigned to the NH of the amide bond, and also present in the above-mentioned pyridyl disulfide-Pluronic, confirmed successful conjugation (see Supporting Information). In Figure 4 the number of pyridyl disulfide groups relative to the number of propylene glycol repeating units on the Pluronic

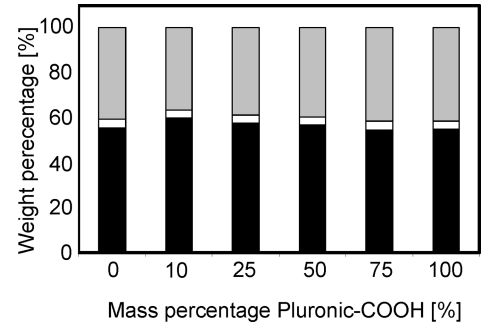


Figure 3. Mass percentage of Pluronic (black), poly(propylene sulfide) (gray), and initiator (white) of total NP weight as a function of weight percentage carboxylate-Pluronic in the polymerization mixture as calculated from NMR.

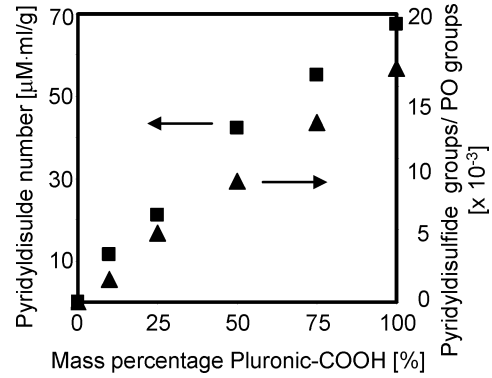
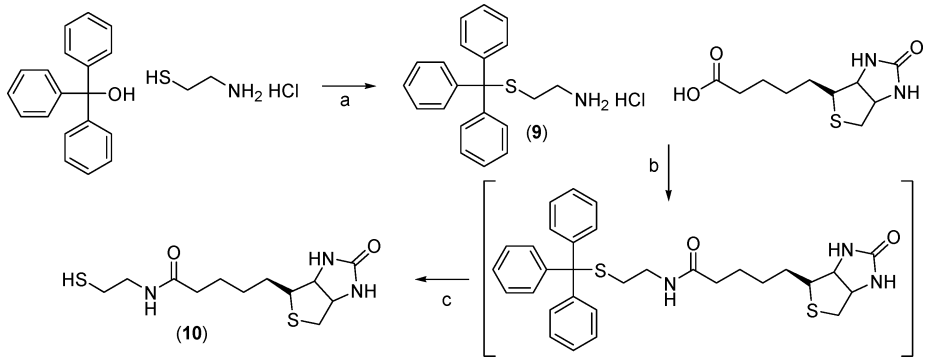


Figure 4. Number of pyridyl disulfide groups divided by the number of repeating propylene oxide (PO) units (triangles) as calculated from NMR from the ratio of pyridyl disulfide groups in μM (as measured by release of pyridine thione after reduction with TCEP·HCl) and NP concentration in mg/mL. The pyridyl disulfide number (squares) is a linear function of weight percentage carboxylate-Pluronic in the polymerization mixture.

as measured with NMR shows that there is a linear relationship between the feed ratio of carboxylate-Pluronic and the amount of pyridyl disulfide-Pluronic present on the NP. However, the reaction does not lead to full conversion of all carboxylate into pyridyl disulfide groups, as can be calculated from the carboxylate-Pluronic feed ratio at 100%. By comparison of the experimentally determined ratio of pyridyl disulfide groups to propylene glycol groups with that theoretically calculated, about 57% of carboxylate groups were determined to have reacted. A similar value of 61% is obtained by measuring the release of pyridine thione after reduction with TCEP·HCl and comparing this concentration with that calculated from the NP concentration and weight percentage of Pluronic in the NP solution. This low functionalization degree is not due to loss and/or modification of carboxylic acid groups during NP synthesis. Control experiments with the carboxylate-Pluronic under conditions used for

Scheme 4. Synthesis of Thiolated Biotin[a]



[a] (a) $BF_3 \cdot Et_2O$, DMF, 80°C, 16 h; (b) EDC, NHS, Et_3N, 2 days; (c) CF_3COOH/TIS, CH_2Cl_2, 4 h.

Table 2. Dynamic Light Scattering Results for the Different Conjugated NPs[a]

compd	*Z*-Avg[b] (SD)	PDI[c] (SD)
biotin	36.4 (0.26)	0.201 (0.003)
peptide	36.7 (0.21)	0.209 (0.003)
OVA	56.0 (0.32)	0.267 (0.006)

[a] Values are the average of three measurements of the same sample. [b] Size in nm as measured by the *Z*-average obtained by fitting the correlation function using the cumulant method. [c] Polydispersity index (PDI) calculated from fitting the correlation function using the cumulant method.

polymerization but without the addition of propylene sulfide did not lead to changes in the NMR spectrum (data not shown). The same linear trend is observed if the total pyridyl disulfide loading in μM is normalized by the NP concentration in mg/mL, the pyridyl disulfide number (Figure 4). Both DLS and TEM confirmed that the NPs kept their small size but that some aggregation occurred as shown by the increase in PDI (Table 1) and the presence of bigger particles in TEM (Figure 1C).

With the pyridyl disulfide-NPs at hand, we wanted to show that we could easily conjugate small molecules, peptides, and proteins. We first considered conjugation of a thiol-containing biotin to the NPs. In Scheme 4, the synthesis of this compound is outlined. After purification by HPLC, the biotin cysteamine was conjugated to pyridyl disulfide-NPs and purified by dialysis. DLS (Table 2) and TEM (Figure 1D) both show that the NPs kept their size. Successful conjugation was shown by adding the biotin-NPs to streptavidin Alexa488 and loading the NP solutions on a SDS−PAGE gel. From Figure 5 (lanes 4−6) it can be seen that above a certain biotin/streptavidin molar ratio, all streptavidin was bound to the NP surface and was no longer able to move into the gel. The reductive sensitivity of the biotin-NP disulfide linkage is clearly illustrated by running the gel (lanes 8−10) in the presence of mercaptoethanol, a reducing agent that leads to cleavage of the disulfide bond between biotin and the NP.

To see the effect of molecular size on conjugation, a model peptide was synthesized with a cysteine added at the C-terminus. Conjugation of the peptide was done at pH 4, and excess peptide was removed by gel filtration on Sepharose 6B beads. Figure 6A shows the elution profile for pyridyl disulfide-NP after reducing the fractions with TCEP·HCl and measuring the absorbance at 340 nm due to pyridinethione release. As can be seen from Figure 6A, the NPs elute as a sharp peak and DLS confirmed the presence of NPs in the fractions. Figure 6B shows the elution profile of peptide-NP after gel filtration as measured after reaction of the lysine residues on the peptide with fluorescamine and measuring the fluorescence intensity. As can be seen, the peptide is bound to the NPs, as the elution profile is practically the same as that measured for the pyridyl disulfide-

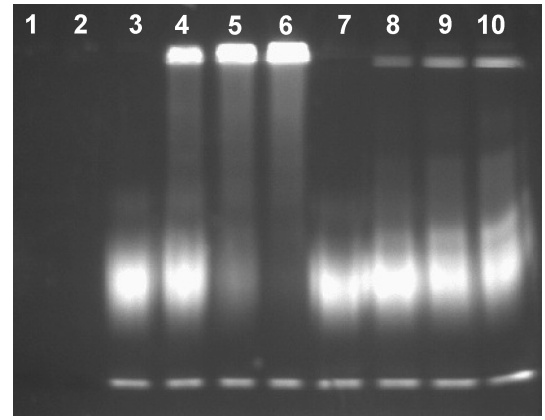


Figure 5. SDS−PAGE gel showing the interaction between biotin-NPs and streptavidin-Alexa488: (lane 1) ladder; (lane 2) biotin-NPs (15 μL); (lane 3) streptavidin-Alexa488 (10 μg); (lanes 4−6) streptavidin-Alexa488 (10 μg) with increasing volumes (5, 10, and 15 μL) of biotin-NPs; (lanes 7−10) same as for lanes 3−6 but in the presence of β-mercaptoethanol.

NP. DLS (Table 2) shows that the NPs still have similar size relative to the pyridyl disulfide-NP. To show that this peptide is linked by a disulfide bond, the peptide-NPs were reduced with TCEP·HCl prior to gel filtration. In Figure 6C it can be seen that the peak in Figure 5B is completely gone and that a new peak can be observed at higher elution volume, which is due to free peptide.

Having shown that a small molecule and a larger peptide can be conjugated to the pyridyl disulfide-NPs, we were also interested in conjugating proteins. We choose OVA, a 44 000 Da protein that after full reduction has six cysteine residues, and we conjugated it to the NPs. From the elution profile shown in Figure 7A, it can be seen that all OVA has been conjugated. Reduction of OVA is necessary, as shown for the elution profile of the reaction mixture of OVA that had not been reduced prior to conjugation (Figure 7B). Clearly OVA elutes much later than when bound to the NPs.

DISCUSSION

We previously reported the emulsion polymerization of propylene sulfide/Pluronic F127 by anionic ring-opening polymerization (*16*). The presence of the two hydroxyl groups of the Pluronic F127 on the surface of the cross-linked polypropylene sulfide core opens the possibility of introducing biological functionality onto the NPs by modifying the α,ω hydroxyl groups. However, this should be done prior to making the NPs, as commonly used hydroxyl activation chemistries are not compatible with the aqueous solution of the NPs. For this reason, Pluronic F127 has been modified with reactive groups and

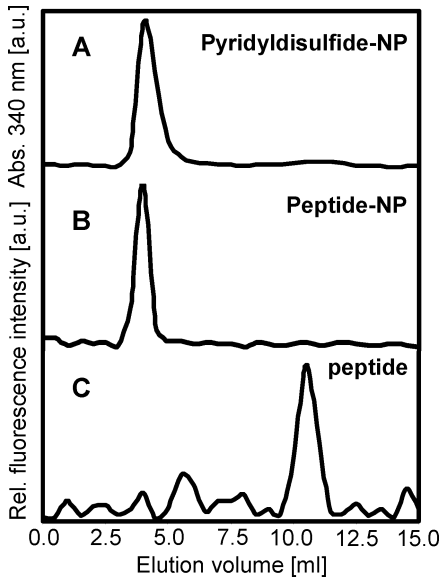


Figure 6. (A) Elution profile of pyridyl disulfide-NPs. Fractions were reduced with TCEP·HCl, and the absorbance at 340 nm due to pyridine thione was measured. (B) Elution profile of peptide-NPs after purification. (C) Elution profile peptide-NPs after reduction with TCEP·HCl. Fractions were reacted with fluorescamine, and the fluorescence intensity was measured.

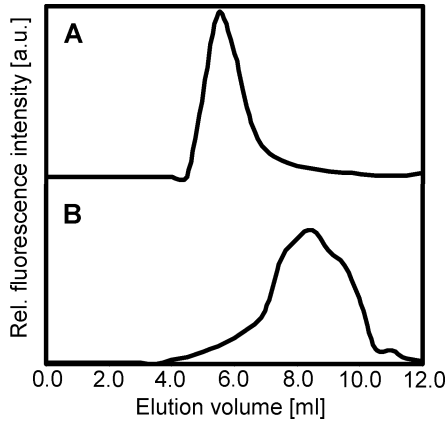


Figure 7. Elution profile after reacting pyridyl disulfide-NPs at pH 8 with (A) reduced and (B) nonreduced ovalbumin. Fractions were analyzed by addition of fluorescamine and measurement of the fluorescence intensity.

reacted with molecules prior to nanoparticle synthesis to obtain biologically functional nanoparticles. Previously we have modified Pluronic F127 with *p*-nitrophenol (*17*) and vinylsulfone (*18*) groups that allow conjugation of peptides (*24*) and proteins (*16, 18*) using amines and thiols.

Despite the successful applications described above, there remains a disadvantage in our previous scheme, in that for making biological-functional NPs each time the corresponding functionalized Pluronic has to be synthesized, which may limit the scope of use of the biological-functional NPs. Furthermore, the polymerization conditions during NP synthesis might be deleterious for the bioactivity of, for example, proteins for which the conservation of sensitive native three-dimensional structure is highly important. Indeed, in the case of the GOx-NPs, despite careful optimization of the synthesis, more than 30% loss of enzyme activity after NP synthesis was observed.

For these reasons, we sought to develop a more flexible system that would allow for mild reaction conditions and broad pH range to conjugate small molecules, peptides, and proteins to the NP surface. We reasoned that a thiol-reactive group on

Chart 1. Commonly Available Thiol-Reactive Groups (A) Vinylsulfone, (B) Acrylate, (C) Maleimide, (D) Alkyl Bromide or Iodide, (E) Pyridyl Disulfide

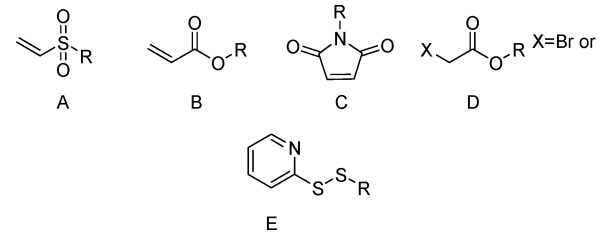
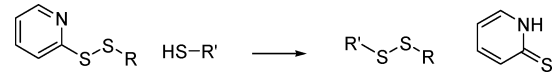


Chart 2. Reaction between Pyridyl Disulfide and a Thiol To Give a Mixed Disulfide with Release of Pyridine Thione



the surface of the NP for binding to the NPs might be most advantageous, as peptides and proteins can be easily engineered to have a free thiol group. In this way with only one NP batch, functionalizable NPs, one would have access to a number of biological-functional NPs.

For the thiol-reactive group on the NP surface, there are several options, as shown in Chart 1. First are the Michael acceptors vinylsulfone (**A**), acrylate (**B**), and maleimide (**C**). These groups react with thiols in a conjugate Michael addition to form a thioether bond between the thiol and double bond. A thioether bond is also formed when the thiol reacts in a nucleophilic substitution reaction with bromo and iodo derivatives (**D**). The disadvantage of these thiol-reactive groups is that a physiologically irreversible thioether bond is formed. In the case of antigen delivery, this might be problematic, as the antigen is not released from the NP after being taken up by the cell unless first proteolytically processed. Despite the fact that the acrylate-based conjugate is cleavable by ester hydrolysis, this group is problematic for long-term storage of the aqueous antigen-NPs solution.

Another thiol-reactive group is the pyridyl disulfide group (**E**) that reacts with thiols to give a disulfide bond and releases pyridine thione as shown in Chart 2. This disulfide exchange reaction is practically irreversible because of the formation of the very stable pyridine thione which has a anomalously low pK_a value (*25*). This is also the reason why this reaction takes place over a broad pH range between 4 and 8 (data not shown). In addition to the broad pH range, conjugation using the pyridyl disulfide group has two other advantages: the amount of protein or peptide conjugated can be quantified by measuring the release of pyridine thione, and the peptide or protein is linked to the NP surface via a reduction-sensitive disulfide bond, which allows a cellular mechanism for antigen release after endocytosis due to the reductive potential early during endolysosomal processing. This increase in reducing environment will result in cleavage of the peptide/protein-NP disulfide bond and allow for release of the peptide or protein from the NP to affect its biological activity. This antigen release mechanism was not possible in the original scheme using the vinylsulfone coupling; rather, proteolytic release was required. For this reason we functionalized the NPs with this pyridyl disulfide group. The most straightforward approach is synthesis of a pyridyl disulfide-Pluronic and adding this to the polymerization mixture to make pyridyl disulfide-NPs. However, a problem is that the thiolate species that are responsible for the polymerization of propylene sulfide could also react with the pyridyl disulfide groups on the Pluronic. Indeed, after synthesizing pyridyl disulfide-Pluronic (*21*) and addition to the polymerization mixture, NMR showed that the pyridyl disulfide group was not present after NP

synthesis (data not shown). To circumvent this problem, i.e., introducing the thiol-reactive pyridyl disulfide group in the presence of thiolate species, we came to the following approach as outlined in Scheme 1. As the first step, carboxylate-functionalized NPs are synthesized and, in the second step, functionalized with a pyridyl disulfide containing a free amine to introduce the thiol reactive groups. Note that we choose the NPs to have a negative charge instead of positive (amines) to avoid the potential toxic effects of cationic charges that could arise from unreacted amine groups. The carboxylate-Pluronic was therefore synthesized in three steps from Pluronic F127 as outlined in Scheme 1. The use of the methyl ester was necessary, as reaction of mercaptopropionic acid also led to substitution at the carboxylic acid leading to an ester bond as was visible from ^{1}H NMR by the occurrence of a triplet around 4.2 ppm. After alkaline hydrolysis, the carboxylate-Pluronic was kept as its sodium salt so as to not interfere during NPs synthesis by lowering the pH. As the final step in characterizing the carboxylate-Pluronic, the polymer was reacted with pyridyl disulfide·HCl to give pyridyl disulfide-Pluronic. This polymer would serve as a reference for characterization of the pyridyl disulfide NPs.

With the carboxylate-Pluronic at hand, NPs were synthesized by emulsion polymerization with different COOH-to-OH Pluronic mass ratios. After exposure to air for 2 h to form disulfides, the remaining free thiolate anions that were spatially too far apart to form disulfides were blocked by the addition of iodoacetamide. Previous work had shown that this is effective in blocking thiolates (*15*) and capping was quantitative as measured by Ellman assay. This blocking is important to avoid a disulfide exchange reaction when the pyridyl disulfide groups are introduced onto the NPs. From the DLS and TEM data, it can be seen that the addition of carboxylate-Pluronic did not significantly affect the size of the final NPs. The PDI, however, dropped significantly (Table 1), suggesting that the negatively charged NP surface stabilizes the particles, which can be explained by DLVO theory, which states that like charge stabilizes colloid particles (*26*). In addition, the carboxylate-Pluronic did not influence the ring-opening polymerization of propylene sulfide to a significant extent as shown by the degree of polymerization and weight composition of the NPs. This shows that the NP synthesis is not affected significantly by the presence of the carboxylate-Pluronic in the polymerization mixture. Because of overlap of the characteristic signals of the carboxylate-Pluronic at 2.5, 2.7, and 2.8 ppm with the broad CH and CH_2 signals at 2.5−2.7 and 2.8−3.0 ppm of the PPS, it was not possible to determine the percentage of carboxylate-Pluronic present in the 10−75% carboxylate-NPs by NMR. For this reason we used the conjugation with pyridyl disulfide cysteamine·HCl as an indirect measurement to obtain insight into how the carboxylate-Pluronic was incorporated into NP surface as a function of the feed ratio in the polymerization mixture. From the 100% carboxylate-NPs, it was calculated that only about 60% functionalization was achieved, even though the carboxylate-Pluronic alone could be functionalized almost quantitatively. This lower degree of functionalization is not due to loss of carboxylate groups as was shown in control experiments. An explanation could be that some carboxylate groups remain inaccessible during functionalization, though how that would happen is unclear: interaction of the negatively charged carboxylate group with the hydrophobic PPS is unlikely. It is therefore more likely that the EDC/sulfo-NHS coupling chemistry itself limits the degree of functionalization because of its inherent water sensitivity. Indeed model experiments in which pyridyl disulfide cysteamine·HCl was coupled (using NHS) to the carboxylate-Pluronic that had been used in NP synthesis led to almost quantitative conversions in CH_2Cl_2 but only 45% in MES (aq) buffer (data not shown). The linear trend in Figure 4 suggests that the percentage of carboxylate-Pluronic on the NP surface is the same as that in the polymerizing mixture but that only about 60% can be functionalized. This finding enables the control of surface density of pyridyl disulfide groups on the NP surface by adjusting the feed ratio in the polymerization mixture of carboxylate- to hydroxyl-Pluronic.

Having shown that pyridyl disulfide groups could be introduced onto the NPs, it was important to show that these are actually in contact with the aqueous phase and available for conjugating with peptides and proteins. To explore this, we synthesized a thiol-containing biotin molecule and reacted it with the pyridyl disulfide-NPs. With this biotin molecule at both ends of the Pluronic, it would also be possible to see whether after conjugating biotin on the Pluronic ends the NP surface would be still accessible for interaction with large proteins. Biotin interacts with streptavidin, a 66 000 Da protein, to form the strongest noncovalent bond known in nature. Indeed biotin on the NPs is able to interact with the binding pockets of streptavidin, as shown in Figure 5. This result shows that the pyridyl disulfide groups on the NP surface are accessible in the aqueous phase for conjugation and that streptavidin is able to interact with the conjugated biotin on the NP surface. The biotin-NPs are furthermore interesting from a staining point of view. Having biotin bound to the NPs enables us the ability to probe the presence of the NPs in tissues by cryosectioning and staining with fluorescently labeled streptavidin.

For immunotherapy applications normally whole immunogenic proteins are used; however, they typically lead to release of a limited number of antigenic peptides when processed within DCs. For example the OVA protein yields two (SIINFEKL and ISQAVHAAHAEINE) antigenic peptide sequences upon processing by DCs (*27*). Since peptides are easily accessible by solid phase peptide synthesis, they can be made to have a free cysteine residue, enabling conjugation to NPs. As a model, we synthesized a nine amino acid peptide containing a cysteine and conjugated it to the NP. For purification of the peptide-NP, gel filtration was used because it is time saving and a better purification method compared to dialysis, especially with increasing size of the molecule to be conjugated. The presence of the peptide on the NPs was confirmed with DLS and fluorescamine (*20*). Important in our concept of antigen-bearing NPs is the reductive sensitivity of the antigen-NP disulfide bond. For this reason, we exposed both the biotin-NPs and peptide-NPs to a reducing agent to demonstrate sensitivity to reduction. In the case of biotin, mercaptoethanol led to a cleavage of the disulfide bond at room temperature as shown by SDS−PAGE gel where the streptavidin was no longer bound to the NP surface and freely moved into the gel. For the peptide-NP, addition of TCEP·HCl also led to disulfide cleavage as could be shown after gel filtration.

Finally we wanted to show that it is also possible to conjugate a larger protein. We choose OVA because it is a model antigen that is used frequently in immunotherapy studies. OVA has four free cysteine residues and one disulfide bond that would allow easy conjugation using our functionalization scheme. The problem is that these four cysteine residues are buried in the native state and are not reactive toward iodoacetic acid at pH 8.2, an alkylation agent (*28*). For this reason we reduced OVA to expose all six cysteine residues (four free and two in a disulfide). This, however, leads to a potential problem of cross-linking, as the NP surface is decorated with several pyridyl disulfide groups. With careful optimization, we managed to conjugate OVA to pyridyl disulfide-NPs. From Figure 6A it can be seen that all OVA eluted in one peak and that free OVA was not detected. DLS indicated an increase in size but the OVA nanoparticles still remained small (Table 2). That practically

no free OVA was present is clearly illustrated in Figure 6B by elution nonreduced OVA, which elutes at a much higher elution volume and shows the need for reducing OVA prior to conjugation. Indeed, if conjugation of reduced OVA is not quantitative, the elution profile shows both peaks (data not shown).

To conclude, we have presented a scheme by which to make thiol-reactive Pluronic-stabilized poly(propylene sulfide) NPs by incorporating a carboxylate-Pluronic into the hydroxyl-Pluronic/propylene sulfide mixture that was subsequently reacted with pyridyl disulfide cysteamine to yield pyridyl disulfide-NPs. The polymerization and NP composition were not affected by the presence of the carboxylate-Pluronic, and the number of pyridyl disulfide groups on the NP surface could be controlled by changing the weight ratio of carboxylate-Pluronic to hydroxyl-Pluronic in the mixture. We further showed that we could conjugate biotin, a nine amino acid peptide, as well as the larger protein OVA. Currently we are exploring the functionalizable NPs scheme for immunological application by conjugating antigenic peptides, proteins, and protein structures with adjuvant activity.

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Supporting Information Available: Calculation of NP composition, NMR spectra of **6** and pyridyl disulfide-NP, and estimation of numbers of biotin, peptide, and OVA molecules per NP. This material is available free of charge via the Internet at http://pubs.acs.org.

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