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Disulfide-Crosslinked Polyion Micelles for Delivery of Protein Therapeutics

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Abstract—New protein delivery systems are needed that efficiently encapsulate proteins and avoid formulation processes that affect protein structure and function. We have developed a protein delivery system termed disulfide-crosslinked polyion micelles (DCPMs), which consist of nanocomplexes formed by electrostatic self-assembly of a protein with a poly(ethylene glycol)-poly(L-lysine) block copolymer (PEG-PLL). The PEG-PLL amines are modified with crosslinkable dithiopyridine groups, using a Michael addition reaction that preserves the positive charges on the PLL chain to optimize polyionic complexation and disulfide crosslinking. DCPMs for vaccine delivery were prepared with ovalbumin and immunostimulatory CpG-DNA and are designed to release the vaccine intracellularly through reduction of disulfide crosslinks. DCPMs were also developed as a long-circulating enzyme carrier that maintains the enzymatic activity of the anti-oxidant enzyme catalase within the micelle core. Ovalbumin and catalase were each modified with SPDP to tether the protein in the micelle core, resulting in a high degree of protein retention under SDS-PAGE. DCPMs efficiently encapsulate and retain functional proteins in a stable polyionic complex and are a versatile delivery system for enzymes, vaccine antigens, and other protein therapeutics.

Keywords—Drug delivery, Nanocomplex, Polylysine, Ovalbumin, Catalase, CpG-DNA.

ABBREVIATIONS

DCPM	Disulfide-crosslinked polyion micelle
PEG-PLL	Poly(ethylene glycol)-*block*-poly (L-lysine)
PEG-PLDTP	Poly(ethylene glycol)-*block*-poly (L-lysine-dithiopyridine)
DODT	3,6-Dioxa-1,8-octanedithiol
PDTEA	Pyridyldithioethylacrylate
DTP	Dithiopyridine

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INTRODUCTION

Therapeutics based upon proteins are an exciting new class of drugs that have the potential to significantly improve the treatment of a variety of human diseases. Protein-based pharmaceutical products have been marketed for cancer treatment, hormone therapy, enzyme therapy, and vaccines, and there are many new protein drugs under investigation.[1–3,8,12,24] The pharmacokinetic properties of protein drugs, however, are often adversely affected by denaturation and enzymatic degradation and their inability to cross cell membranes.[3,5] Therefore, the efficacy of protein therapeutics can be improved through the use of a drug delivery system that maintains the chemical and physical stability of the protein and overcomes transport barriers. In particular, protein delivery vehicles can improve the blood circulation half-life of proteins and target proteins to the desired site of action.

Several protein delivery vehicles have been developed, such as microparticles, microgels, and block copolymer micelles.[11,15,23] Polymeric microparticles, composed of poly(lactic-*co*-glycolic acid) (PLGA), are the most well established protein delivery vehicle, and have had clinical applications.[25,27] Although PLGA microparticles can generate a sustained release of protein therapeutics after a single injection, there are several inherent disadvantages with polymeric microparticles that have limited their ability to deliver proteins. A primary concern with PLGA microparticles is that protein encapsulation requires exposing the protein therapeutic to organic solvents, which frequently results in protein denaturation and loss of protein function.[29] A second limitation of polymeric microparticles is that microparticle batches cannot be efficiently prepared on a small scale, making it

economically prohibitive to conduct studies with expensive recombinant proteins. Finally, with formulations containing low levels of recombinant protein, it is difficult to quantify the amount of encapsulated protein using bulk protein assays, thus necessitating the use of functional protein assays. This is problematic with microparticles because the methods of extracting proteins may result in inactivation of the encapsulated protein.[26,29] Therefore, new protein delivery vehicles are needed to overcome these limitations.

Protein delivery vehicles based upon polyion complex micelles have great promise for enhancing the efficacy of protein therapeutics because of their ability to encapsulate proteins in aqueous solutions and their ability to encapsulate small quantities of biomolecules over a wide range of batch sizes. Polyion micelles are formulated by self-assembling a poly(ethylene glycol) (PEG) block copolymer, which contains a charged segment, with an oppositely charged protein. This produces core–shell micelles, which have a core formed by electrostatic interactions between the charged biomolecule and the polyion block, while the PEG chain occupies the corona of the micelle.[10,11] Polyion micelles generally require a method for stabilizing the micelles against decomposition induced by serum proteins or high salt concentrations.[32] The stability of polyion micelles has been improved by introducing hydrophobic groups in the core-forming block or by cross-linking the protein or polymer.[14,16,32] For example, micelles composed of PEG-poly(aspartic acid) and trypsin or lysozyme were core cross-linked using glutaraldehyde, which forms imines through a Schiff base reaction with primary amines of the protein. This method, however, has the disadvantages of multiple potential side reactions,[6] potential toxicity of unreacted glutaraldehyde, and the inability of directly monitoring the cross-linking reaction. Glutaraldehyde is the only reported cross-linking scheme for protein-containing polyion micelles, and thus new approaches for cross-linking protein-loaded polyion micelles are greatly needed.

In this report, we describe a new method of cross-linking proteins in polyion micelles using the disulfide exchange reaction, generating a stable but degradable protein delivery vehicle. The general design of the disulfide-crosslinked polyion micelle (DCPM) is shown in Fig. 1. The block copolymer PEG-poly(L-lysine-dithiopyridine) (PEG-PLDTP) forms the scaffold of the DCPM and is composed of PEG-poly(L-lysine) (PEG-PLL) grafted with dithiopyridine groups. The DCPMs are formed via self-assembly of PEG-PLDTP with proteins bearing a net negative charge. The resulting core–shell micelles are then cross-linked with a dithiol, generating DCPMs. Importantly, disulfide cross-links

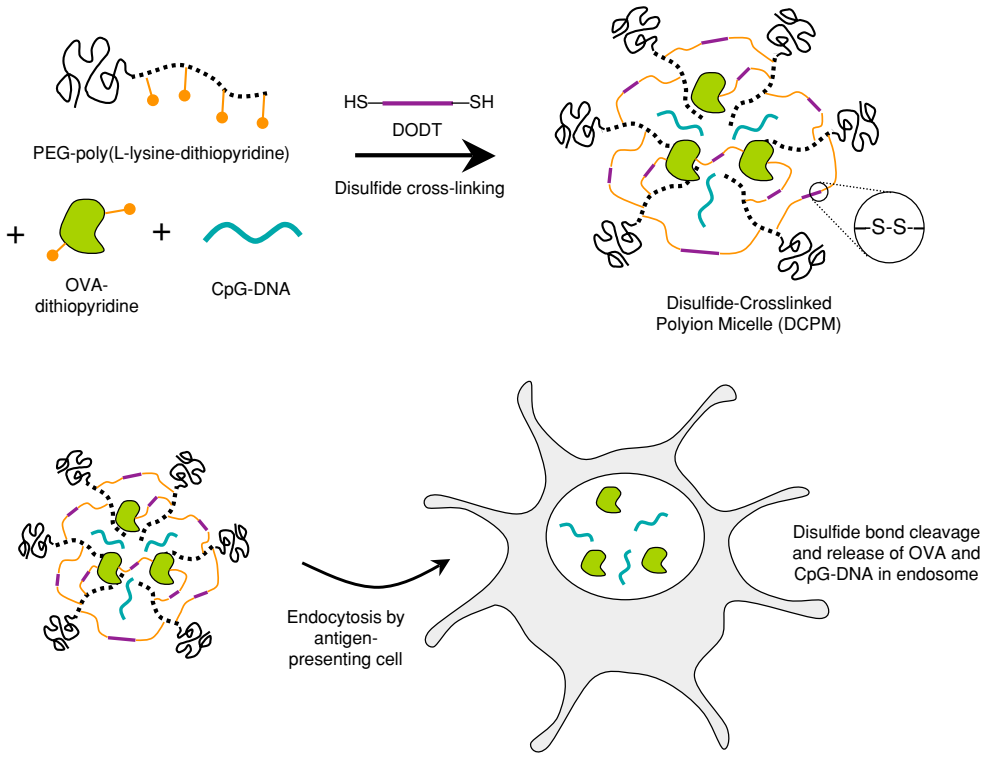

FIGURE 1. Design strategy of disulfide-crosslinked polyion micelle (DCPM). (a) Polyion complex micelle is formed by self-assembly of PEG-PLDTP, OVA, and CpG-DNA and is disulfide cross-linked to enhance stability; (b) DCPM is endocytosed by antigen-presenting cell and dissociates upon reduction of disulfide bonds, leading to intracellular release of OVA and CpG-DNA.

are relatively stable in serum and the extracellular fluid, but are cleaved under intracellular reducing conditions, thereby providing a versatile platform for delivery of therapeutic proteins.[9,16] Two different types of protein therapeutics were encapsulated in DCPMs. A model vaccine formulation was synthesized using DCPMs containing the antigen ovalbumin (OVA) and immunostimulatory CpG-DNA (OVA-CpG-DCPMs). OVA-CpG-DCPMs are designed to deliver protein antigen and CpG-DNA to phagosomes of antigen-presenting cells (APCs), where CpG-DNA activates the APC via Toll-like receptor 9 and the protein is processed for cross-presentation to T lymphocytes. A second type of DCPMs was also synthesized, which contained the anti-inflammatory enzyme catalase (CAT). To provide greater stability to the DCPMs, OVA and CAT were each modified with dithiopyridine moieties to tether the protein in the micelle core during the cross-linking step. We investigated the ability of the DCPMs to encapsulate and retain proteins in denaturing gel electrophoresis (SDS-PAGE), and compared the enzymatic activity of CAT-DCPMs to free catalase to determine if encapsulation in the DCPMs influenced the enzymatic function of catalase.

MATERIALS AND METHODS

Materials

Triphosgene, dithiothreitol (DTT), albumin from chicken egg white (A5503, ovalbumin (OVA)), 3,6-dioxa-1,8-octanedithiol (DODT), and 2,2-dithiopyridine (Aldrithiol) were purchased from Sigma-Aldrich (St. Louis, MO). Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP) was purchased from Pierce Protein Research Products (Rockford, IL). *N*-ε-Benzyloxycarbonyl-L-lysine (H-Lysine(Z)-OH) was sourced from Bachem (Torrance, CA) and Novabiochem (San Diego, CA). Fluorescein-5-isothiocyanate (FITC 'Isomer I') was purchased from Invitrogen (Carlsbad, CA), and α-methoxy-ω-amino-poly(ethylene glycol) (PEG-NH_2, MW 5000) was obtained from Nektar Therapeutics (Huntsville, AL). Catalase from bovine liver (CAT, product number 219001) was obtained from Calbiochem (Gibbstown, NJ). CpG-DNA oligonucleotide (5′-TCCATGACGTTCCTGACGTT-3′) was purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Synthesis of Poly(Ethylene Glycol)-Poly(L-Lysine) (PEG-PLL) Block Copolymer

PEG-PLL was synthesized by ring opening polymerization of PEG-NH_2 with the *N*-carboxyanhydride (NCA) of *N*-ε-benzyloxycarbonyl-L-lysine (Lys(Z)-NCA).[10,16] H-Lys(Z)-OH (10 g, 35.7 mmol) was reacted with triphosgene (4.9 g, 16.6 mmol) for 4 h at 50 °C in dry THF. The product was recrystallized three times in cold hexanes to produce Lys(Z)-NCA with a 72% yield. The poly(ethylene glycol)-*block*-poly(L-lysine(Z)) (PEG-PLL(Z)) copolymer was synthesized by reacting PEG-NH_2 (0.5 g, 0.1 mmol) with Lys(Z)-NCA (1.84 g, 6.0 mmol) for 20 h at 40 °C in anhydrous DMF. The product was precipitated in cold ethyl ether, vacuum filtered, and vacuum dried to yield 937 mg of PEG-PLL(Z) block copolymer. From the ^{1}H NMR spectrum, the degree of polymerization (d.p.) was calculated to be 31.8. Deprotection of the ε-benzyloxycarbonyl group was carried out by mixing 800 mg of PEG-PLL(Z) with 8 mL of trifluoroacetic acid, then with a mixture of 8 mL of anisole and 5.6 mL of methanesulfonic acid, followed by ether/water extraction. The aqueous phase was dialyzed against a 1000 molecular weight cut-off membrane, and lyophilized to yield 254 mg of PEG-PLL. After deprotection, the d.p. was calculated to be 10.2 based on the ^{1}H NMR spectrum.

Synthesis of Pyridyldithioethylacrylate (PDTEA)

Mercaptoethanol (0.886 g, 11.36 mmol) was reacted with 2,2-dithiodipyridine (5 g, 22.7 mmol) and acetic acid (1.36 g, 22.7 mmol) in 15 mL of methanol for 1 h at room temperature. The 2-(2-pyridyldithio)-ethanol product was purified by silica gel chromatography with 79.5% yield. Next, 2-(2-pyridyldithio)-ethanol (1.6 g, 8.56 mmol) was dissolved in 16 mL of dichloromethane (DCM) with triethylamine (1.297 g, 12.84 mmol). Acryloyl chloride (1.156 g, 12.84 mmol) was added in a dropwise manner to initiate the reaction. After 1 h, the product was purified by DCM/brine extraction (2 times), dried with sodium sulfate, and purified by silica gel chromatography, for a 64% yield of PDTEA.

Synthesis of PEG-Poly(Lysine-Dithiopyridine) (PEG-PLDTP) Copolymer

PEG-PLDTP was synthesized by grafting PDTEA to PEG-PLL via a Michael addition reaction.[9] Briefly, PEG-PLL (124 mg) was dissolved in 4 mL of DMF, and PDTEA (62.5 mg, 260 μmol) was added with an additional 1 mL of DMF. Triethylamine (69 μL, 495 μmol) was added, and the reaction was run at 30 °C overnight. The reaction mixture was precipitated in 125 mL of cold ethyl ether, vacuum filtered, and vacuum dried to yield 112 mg of polymer. The final polymer is termed poly(ethylene glycol)-*block*-poly(L-lysine-*N*-pyridyldithioethylethanoate), or PEG-poly(lysine-dithiopyridine) (PEG-PLDTP). Based on

^{1}H-NMR measurements of the pyridine protons vs. the α,β,γ-methylene protons of PLL, 80–100% of the amines were reacted.

Preparation of SPDP-FITC-OVA and SPDP-FITC-CAT

To prepare SPDP-FITC-OVA, a 6.0 mg/mL solution of FITC-labeled OVA (15 mg, 0.34 μmol) in pH 7.2 buffer was reacted with Sulfo-LC-SPDP (2.15 mg, 4.08 μmol) and purified by PD-10 column, resulting in a degree of modification of 5.45 dithiopyridine groups per protein molecule. To prepare SPDP-FITC-CAT, a 5.0 mg/mL solution of FITC-labeled catalase (12.5 mg, 0.209 μmol) was reacted with Sulfo-LC-SPDP (0.882 mg, 1.67 μmol) and purified by PD-10 column, resulting in 2.23 dithiopyridine groups per catalase monomer.

The loss of enzymatic activity due to FITC and SPDP modifications was determined using a hydrogen peroxide conversion assay. Solutions of catalase, FITC-CAT, and SPDP-FITC-CAT were prepared at approximately 2.5 μg/mL in 50 mM potassium phosphate buffer (KP50), and then diluted 12.5-fold into 20 mM hydrogen peroxide in KP50 to initiate the enzymatic reaction. The decrease in absorbance at 240 nm was measured over a 120 s window to calculate the catalase activity. FITC modification resulted in approximately 25% loss of catalase activity, and sulfo-LC-SPDP modification did not affect activity.

Preparation of DCPMs

The block copolymer PEG-PLDTP was used to prepare DCPMs containing proteins and nucleic acids. DCPMs were prepared by first combining solutions of SPDP-FITC-OVA (100 μg in 35 μL) and CpG-DNA (100 μg in 10 μL), and vortexing to ensure thorough mixing. Next, a solution of the PEG-PLDTP copolymer (2.0 mg in 100 μL) was added to form the micelles. Alternatively, OVA-DCPMs were prepared with SPDP-FITC-OVA and PEG-PLDTP, and CAT-DCPMs were prepared with SPDP-FITC-CAT (100 μg in 23.4 μL) and PEG-PLDTP (2 mg in 100 μL). The mixtures were left to stand at room temperature for 30 min to allow self-assembly of the micelles. Next, DODT was added in multiple stages to cross-link the dithiopyridine groups through a disulfide exchange reaction. The micelles were allowed to stand for 30 min following each addition of DODT to complete the disulfide exchange, and afterwards the total volume was adjusted to 200 μL with deionized water.

The molar ratio of DODT thiols to dithiopyridine groups was between 1.05 and 1.10. The molar amount of dithiopyridines on PEG-PLDTP was calibrated prior to micelle preparation by adding DTT and measuring 2-thiopyridone formation at 342 nm. DCPMs were also tested with DTT to verify conversion of dithiopyridines, which was typically 98–100%. The amount of free thiols remaining after cross-linking was calculated to be 5–12% of the initial DODT thiols; thus, the free thiol content of the DCPMs was typically less than 0.4 mM.

Characterization of DCPMs

DCPMs were sized by dynamic light scattering (DLS), using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY). OVA-CpG-DCPMs were diluted 10-fold in purified water and filtered through a 0.45 μm Acrodisc® syringe filter (Pall Corp, East Hills, NY) prior to DLS measurements. Protein and DNA loading were determined by gel shift assay using SDS-PAGE (4–15% Ready Gel) and agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, CA). Gels were imaged on a Chemi-Doc™ XRS instrument and processed using Quantity One® software (Bio-Rad Laboratories, Hercules, CA). Integrated densities of fluorescent bands (FITC or ethidium bromide) were measured using Image J software (NIH). Micelle morphology and size was analyzed by atomic force microscopy (AFM). Atomic force images were acquired using a Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA) equipped with a J scanner operating in tapping mode. AFM tips were NSC12 non-contact silicon rectangular cantilevers (Mikromasch USA, Portland, OR) which were cleaned with ozone prior to use. DCPMs were dried onto freshly-cleaved mica, and samples were imaged in air under ambient conditions using a 1.5 μm field width. Images were flattened to remove background slope in the horizontal dimensions. Cross sectional analysis of the half-peak widths of representative particles were performed using Nanoscope 5.30 software.

Measurement of Enzymatic Activity of CAT-DCPMs

The enzymatic activity of catalase was determined by the rate of conversion of hydrogen peroxide. CAT-DCPMs and free SPDP-FITC-CAT solutions were prepared at 0.5 mg/mL catalase concentration, diluted 200-fold in 50 mM potassium phosphate buffer (KP50), and then either left unfiltered or filtered through a 0.1 μm Acrodisc® syringe filter (Pall Corp, East Hills, NY). The samples were then diluted 12.5-fold into 20 mM hydrogen peroxide in KP50 to initiate the enzymatic reaction. The decrease in absorbance

at 240 nm was measured over a 120 s window to calculate the catalase activity.

RESULTS AND DISCUSSION

Molecular Design of the DCPMs

In this report we present a new methodology for synthesizing cross-linked protein-loaded micelles. The DCPM strategy overcomes certain limitations of solid microparticle delivery systems by achieving high efficiency encapsulation and avoiding exposure to organic solvents or high shear stresses that can denature proteins and affect their function or immunogenicity. DCPMs are formed by self-assembly of PEG-PLL and a protein carrying a net negative charge. The protein and PLL block form the core of the micelle through electrostatic interactions, while the PEG block occupies the corona, or shell, of the micelle. Because DCPMs are prepared via mixing of aqueous solutions, batches can be scaled to small volumes with minimal loss of yield, making this approach suitable for expensive recombinant proteins.

DCPMs are cross-linked with disulfide linkages to enhance their stability in serum and high ionic strength physiological fluids. Disulfide cross-links are advantageous because they are preferentially cleaved under intracellular reducing conditions, thereby providing a means of intracellular release of vaccines or therapeutic proteins.[9,16] Disulfide cross-linking is achieved by modifying the PEG-PLL backbone with thiol-reactive dithiopyridine (DTP) groups. Following self-assembly of the micelles, a dithiol cross-linker is added to rapidly cross-link the PLL core. The use of DTP chemistry is preferred over other methods involving oxidation of sulfhydryl groups,[21,22] which could potentially disrupt protein disulfide bridges. Also, the DTP reaction proceeds with rapid kinetics and can be monitored *in situ* by 2-thiopyridone formation. The DCPM strategy utilizes a synthetic route developed by the Murthy laboratory to graft the DTP groups onto the PEG-PLL backbone via a Michael addition reaction between PEG-PLL and PDTEA.[9] This synthetic route has the advantage of preserving the positive charge on the lysine side chain, which maximizes the number of cations on the PLL block and facilitates complexation with proteins bearing a net negative charge. As a further modification, we have attached DTP groups to the protein to covalently tether the protein in the core of the micelle. The covalent tethering would be especially useful for proteins having a low charge density or a neutral isoelectric point, in which case the electrostatic interactions and PLL core cross-linking alone may be insufficient to retain the protein.

Synthesis of PEG-PLDTP Block Copolymer

PEG-PLDTP was synthesized in a six-step convergent route, starting from commercially available materials (Fig. 2). The PEG-PLL backbone was synthesized via a ring-opening polymerization of an *N*-carboxyanhydride (NCA) lysine derivative. This monomer was synthesized by reacting a benzyloxycarbonyl (Cbz)-protected lysine (Lys(Z)) with triphosgene to generate the amine-reactive *N*-carboxyanhydride (NCA) group with a 72% yield. The NCA group of the protected lysine was then polymerized, using an amine-terminated PEG as the initiator, to produce PEG-PLL(Z) with a degree of polymerization (d.p.) of 31.8 and 53% yield. The Cbz protecting group was removed from the PLL(Z) side chain with strong acid, followed by dialysis to yield PEG-PLL with a d.p. of 10.2 (lysine units). The change in d.p. during the deprotection and dialysis steps is attributed to the removal of low molecular weight PLL chains that were initiated by water molecules in the ring-opening polymerization reaction. In a parallel path, PDTEA was synthesized in two steps using mercaptoethanol, 2,2-dithiodipyridine, and acryloyl chloride, with purification at each step by silica gel chromatography. This generated a heterobifunctional molecule with an amine-reactive acrylate group and a thiol-reactive dithiopyridine group. The final step was to graft PDTEA to PEG-PLL via a Michael addition reaction between the acrylate and PLL amines, which proceeded with nearly quantitative yield, resulting in conversion of 80 to 100% of the lysine amines.

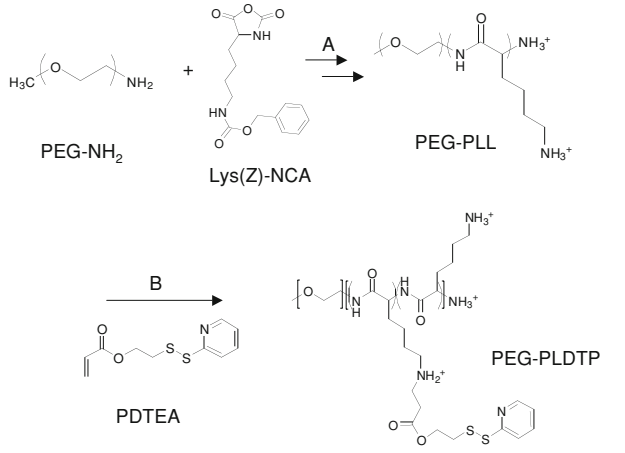

FIGURE 2. Synthesis of poly(ethylene glycol)-*block*-poly (L-lysine-dithiopyridine) (PEG-PLDTP). (A) Ring-opening polymerization of PEG-NH_2 with the *N*-carboxyanhydride (NCA) of Cbz-protected lysine (Lys(Z)-NCA) to generate PEG-poly (L-lysine) (PEG-PLL). (B) Grafting of pyridyldithioethylacrylate (PDTEA) onto PEG-PLL via a Michael addition reaction to generate PEG-PLDTP.

Preparation of DCPM Vaccine Delivery System for Protein Antigen and Immunostimulatory CpG-DNA

We have developed DCPMs as a vaccine delivery system, containing OVA as a model protein antigen and CpG-DNA as an immunostimulatory agent. CpG-DNA is an oligonucleotide with cytidine-guanosine motifs found in bacterial DNA and is an agonist for Toll-like receptor (TLR)9, a membrane-bound endosomal receptor.[19,30] The OVA-CpG-DCPMs are designed to be endocytosed by APCs, where intracellular reducing agents such as glutathione will cleave the disulfide cross-links, resulting in release of protein antigen and CpG-DNA. Ligation of TLR9 by CpG-DNA provides a "danger signal" to the APC, which leads to secretion of costimulatory cytokines and cross-priming of T lymphocytes.[18,19,30] The DCPMs are thus designed to induce a T lymphocyte response by co-delivery of protein antigen and TLR9 agonist to APCs.

To prepare OVA-CpG-DCPMs, a mixture of SPDP-FITC-OVA and CpG-DNA was combined with PEG-PLDTP and allowed to self-assemble for 30 min. The cross-linker DODT was added in two stages, with 30 min after each stage. The disulfide exchange reaction between a thiol and a dithiopyridine group proceeds with rapid kinetics and is commonly used in protein conjugations. This reaction can be monitored by formation of the byproduct, 2-thiopyridone, which absorbs strongly at 342 nm. Following the cross-linking step, a sample of the DCPMs was reacted with excess DTT to measure the amount of remaining dithiopyridine groups; typically less than 2% of the dithiopyridine groups remained unreacted. The purpose of adding DODT in multiple stages was to optimize the degree of cross-linking; e.g., adding 10% excess DODT in one step would potentially result in only 90% of the dithiopyridine groups forming a cross-link, due to DODT molecules reacting only at one end.

Characterization of OVA-CpG-DCPMs included DLS and AFM to determine size and morphology, and gel electrophoresis to demonstrate encapsulation and retention of protein and DNA. DLS measurements showed that the OVA-CpG-DCPMs had an effective diameter of 130 nm, with clusters in the size distribution at 45 and 190 nm (Fig. 3). AFM images were consistent with a spherical shape, and measurement of the half-peak widths ranged from 50 to 150 nm (Fig. 4). The multiple size populations of the DCPMs suggest that the micelles have not reached thermodynamic equilibrium prior to the cross-linking step. An SDS-PAGE gel shift assay of OVA-DCPMs and OVA-CpG-DCPMs showed 85–90% encapsulation of SPDP-FITC-OVA (Fig. 5a). The stability of the DCPMs in the presence of SDS is consistent with the high degree of stability reported for peptide-crosslinked micelles in

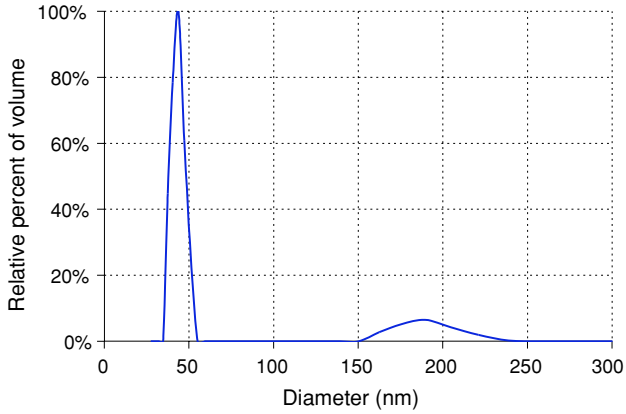

FIGURE 3. Particle sizing of DCPMs containing OVA and CpG-DNA; dynamic light scattering (DLS) multimodal size distribution by volume.

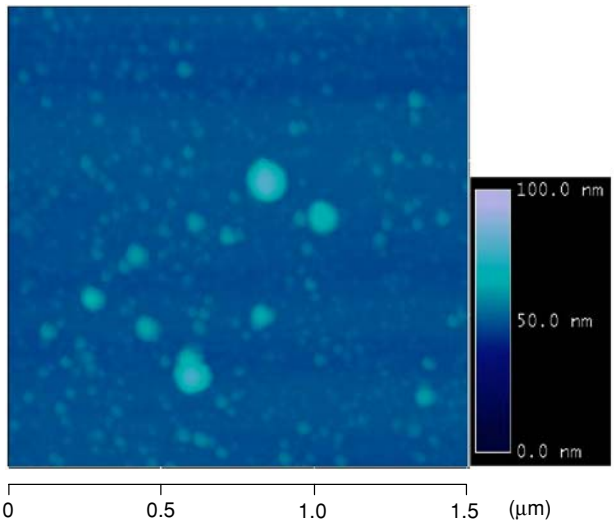

FIGURE 4. Characterization of DCPMs containing OVA and CpG-DNA by atomic force microscopy (AFM).

poly(vinyl sulfate).[9] An agarose gel shift assay of OVA-CpG-DCPMs demonstrated 48% encapsulation of CpG-DNA (Fig. 5b). The lesser retention of CpG-DNA, in comparison to OVA, may be attributed to the fact that CpG-DNA is held in the DCPM core by electrostatic interactions, but is not covalently linked.

The results demonstrate coencapsulation of protein antigen and immunostimulatory CpG-DNA in DCPMs. The disulfide crosslinks provide stability to the micelle and protect CpG-DNA from serum degradation.[9] The disulfide bonds are designed to be cleaved by intracellular reducing agents, thereby delivering antigen and CpG-DNA to the endosomes of APCs, where the CpG-DNA engages TLR9 and the antigen is processed for cross-presentation. Vaccine delivery systems that coencapsulate antigens and adjuvants have been shown to elicit greater T-cell

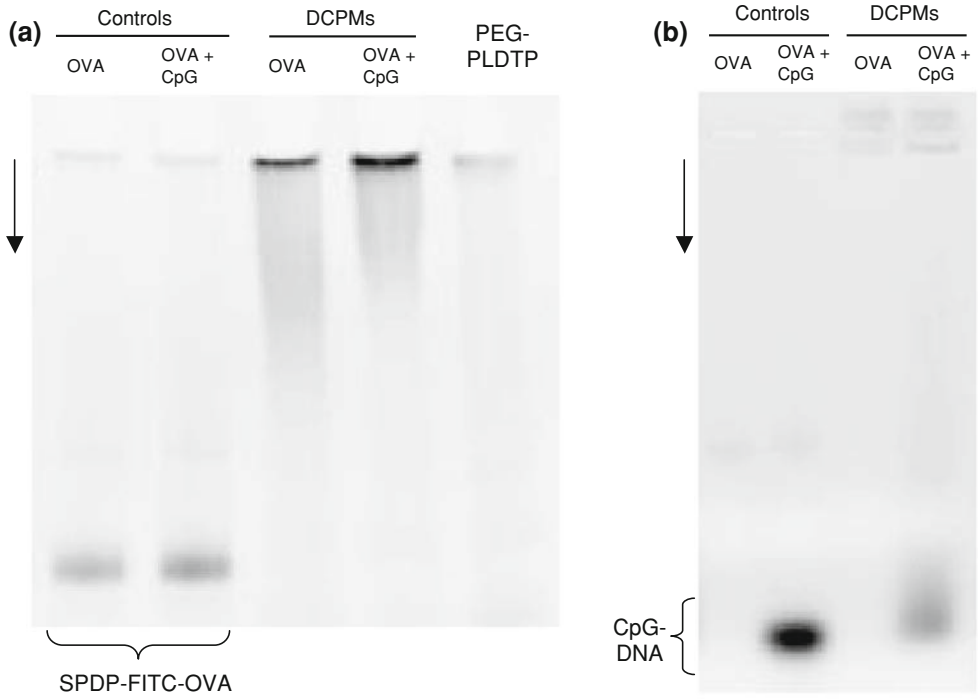

FIGURE 5. Gel shift assays of DCPMs containing OVA and CpG-DNA. (a) SDS-PAGE gel imaged by FITC fluorescence; (b) agarose gel stained with ethidium bromide. DCPMs were prepared with 0.5 mg/mL SPDP-FITC-OVA, 0.5 mg/mL CpG-DNA, and 10 mg/mL PEG-PLDTP.

priming than formulations of soluble antigens and adjuvants.[7,12,28] The DCPMs are an attractive platform for delivering vaccines containing protein antigen and immunostimulatory DNA.

Preparation of a DCPM Enzyme Delivery System

We performed experiments to determine if the DCPMs could also encapsulate enzymes. The antioxidant enzyme catalase was encapsulated in DCPMs and their ability to scavenge hydrogen peroxide was determined. Catalase has great clinical potential because the overproduction of reactive oxygen species has been implicated in a variety of diseases, ranging from cancer to neurodegenerative disease.[4] Catalase-containing DCPMs were prepared via self-assembly of CAT with PEG-PLDTP and cross-linking with DODT. The CAT-DCPMs are designed to be a long-circulating enzyme delivery system for intravenous administration. The PEG corona provides for shielding against rapid clearance by the mononuclear phagocyte system, a technique that has been used with long-circulating micelles and liposomes.[13,17] Disulfide cross-linking provides serum stability to the DCPMs and covalently tethers catalase in the core of the micelle. An important design requirement of the CAT-DCPMs is that the catalase retains its enzymatic activity while entrapped in the micelle core.

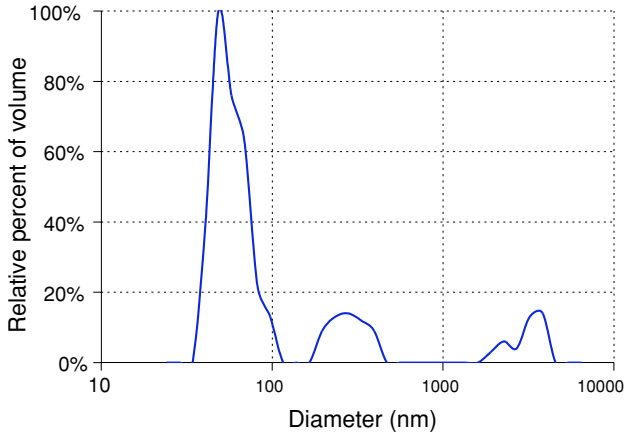

FIGURE 6. Particle sizing of DCPMs containing catalase; dynamic light scattering (DLS) multimodal size distribution by volume. Particle diameter is plotted on a logarithmic scale.

CAT-DCPMs were characterized by DLS, gel shift assays, and enzymatic activity assays to determine their size and their ability to encapsulate functional catalase. DLS measurements of CAT-DCPMs gave an effective diameter ranging from 200 to 300 nm. A representative DLS size distribution by volume is shown in Fig. 6, indicating that the majority of micelles (90% by volume) are in the range from 40 to 400 nm. The SDS-PAGE gel shift assay demonstrates 93% encapsulation of catalase in the DCPMs (Fig. 7).

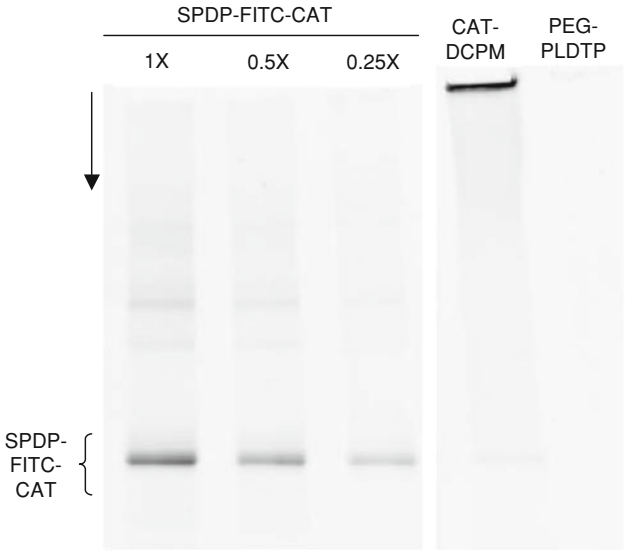

FIGURE 7. SDS-PAGE gel shift assay of DCPMs prepared with 0.50 mg/mL SPDP-FITC-catalase and 10 mg/mL PEG-PLDTP. Control lanes contain 3.75 μg (1×), 1.88 μg (0.5×), and 0.94 μg (0.25×) catalase, and DCPM-CAT lane contains 3.75 μg catalase. Polymer control lane contains 75 μg PEG-PLDTP.

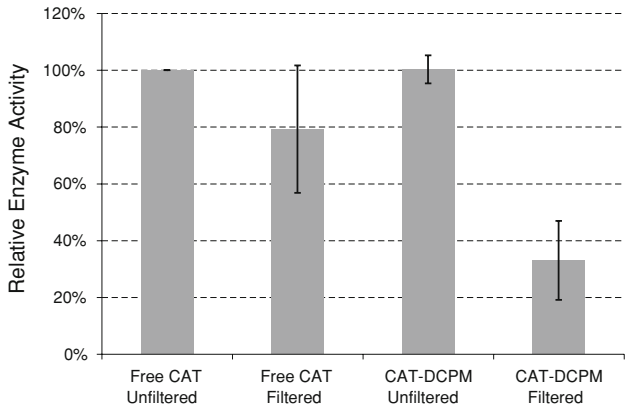

FIGURE 8. Enzyme kinetics of DCPMs prepared with 0.50 mg/mL SPDP-FITC-catalase and 10 mg/mL PEG-PLDTP. CAT-DCPMs were diluted 200-fold and filtered through a 0.1 μm Acrodisc filter or left unfiltered. Samples were diluted 12.5-fold into 20 mM hydrogen peroxide and the decrease in 240 nm absorbance was measured over a 120 s window. Data is plotted as mean ± SD of three independent experiments.

A secondary method of measuring encapsulation was to compare the enzymatic activity of CAT-DCPMs filtered through a 0.1 μm membrane vs. unfiltered CAT-DCPMs. This assay, when adjusted for loss of catalase in the filter, indicates 58% encapsulation of catalase (Fig. 8). The enzymatic assay was also used to show that the catalase activity of unfiltered CAT-DCPMs is equal to the activity of unfiltered free SPDP-FITC-CAT (Fig. 8), indicating that enzymatic function is preserved in the micelle core.

These results demonstrate the synthesis of a stable nanocomplex with properties well suited to enzyme delivery applications. The DCPMs are designed to protect the enzyme from serum degradation by immobilizing the enzyme in the micelle core. The DCPMs have a size range suitable for intravenous injection and utilize PEG shielding for extended circulation. The DCPMs may also be utilized in enzyme-prodrug strategies, by targeting the enzyme to tumors via the enhanced permeability and retention effect.[20,31] We have shown that the enzymatic activity of catalase is preserved while entrapped in the core of the micelle. This is a significant feature, as many microparticle-based delivery systems involve exposure to organic solvents, which can result in denaturation and loss of enzyme function. Based on these demonstrated properties, we expect that the DCPMs will have numerous potential applications in the delivery of catalase and other therapeutic enzymes.

CONCLUSIONS

In this study, we demonstrated the encapsulation of negatively charged proteins in cross-linked DCPMs as a versatile protein delivery system with potential applications in vaccine delivery, treatment of inflammatory diseases, and enzyme delivery. The DCPM efficiently entraps protein molecules via self-assembly with a PEG-PLL block copolymer, generating a polyionic complex with a PEG corona and a core formed by electrostatic interactions. The PLL chain and the protein molecules are modified with dithiopyridine moieties that enable disulfide cross-linking, resulting in a stable complex with a stimulus-responsive intracellular release mechanism. DCPMs were prepared with OVA protein antigen and CpG-DNA as a model vaccine formulation designed to release protein antigen and immunostimulatory CpG-DNA in the endosomes of APCs. Catalase-containing DCPMs were prepared as an antioxidant therapy for treating inflammatory conditions. CAT-DCPMs also represent a long-circulating enzyme delivery system with potential applications in enzyme reconstitution therapy or tumor-targeted enzyme-prodrug therapy.

DCPMs are an attractive delivery system for enzymes and other proteins that are prone to loss of function from exposure to organic solvents during typical microparticle encapsulation procedures. The encapsulation of proteins in DCPMs is done under mild conditions in aqueous solution, without the use of organic solvents. Preservation of protein function in the DCPM core has been demonstrated for the model enzyme catalase. In addition, DCPMs can be prepared under sterile conditions by simple mixing and can be

scaled to small quantities with no apparent loss of efficiency or yield. The DCPM design strategy can potentially accommodate proteins with neutral isoelectric points due to the covalent tethering of the protein in the micelle core. The DCPMs thus represent a versatile platform for long-circulating or intracellular delivery of functional proteins.

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