Title: Diaminosulfide based polymer microparticles as cancer vaccine delivery systems

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Abstract

The aim of the research presented here was to determine the characteristics and immunostimulatory capacity, *in vivo*, of antigen and adjuvant co-loaded into microparticles made from a novel diaminosulfide polymer, poly(4,4'-trimethylenedipiperdyl sulfide) (PNSN), and to assess their potential as cancer vaccine vectors. PNSN microparticles co-loaded with the antigen, ovalbumin (OVA), and adjuvant, CpG 1826, (PNSN(OVA + CpG)) were fabricated and characterized for size (1.64 μm diameter; PDI = 0.62), charge (-23.1 ± 0.3), and loading efficiencies of antigen (7.32 $\mu\text{g}/\text{mg}$ particles) and adjuvant (0.95 $\mu\text{g}/\text{mg}$ particles). The ability of PNSN(OVA + CpG) to stimulate cellular and humoral immune responses *in vivo* was compared with other PNSN microparticle formulations as well as with poly(lactic-co-glycolic acid)(PLGA)-based microparticles, co-loaded with OVA and CpG (PLGA(OVA + CpG)), an adenovirus encoding OVA (Ad5-OVA), and OVA delivered with incomplete Freund's adjuvant (IFA(OVA)). *In vivo* OVA-specific IgG₁ responses, after subcutaneous prime/boosts in mice, were similar when PNSN(OVA + CpG) and PLGA(OVA + CpG) were compared and the presence of CpG 1826 within the PNSN microparticles demonstrated significantly improved responses when compared to PNSN microparticles loaded with OVA alone (PNSN(OVA)), plus or minus soluble CpG 1826. Cellular immune responses to all particle-based vaccine formulations ranged from being negligible to modest with PNSN(OVA + CpG) generating the greatest responses, displaying significantly increased levels of OVA-specific CD8⁺ T lymphocytes compared to controls and IFA(OVA) treated mice. Finally, it was shown that of all vaccination formulations tested PNSN(OVA + CpG) was the most protective against subsequent challenge with an OVA-expressing tumor cell line, E.G7. Thus, microparticles made from poly(diaminosulfide)-based macromolecules possess promising potential as vaccine vectors and, as demonstrated here, may have impact as cancer vaccines in particular.

KEYWORDS: poly(4,4'-trimethylenedipiperdyl sulfide); cancer vaccine; microparticles; PNSN; nanoparticles, biodegradable polymer, antigen; CpG, PLGA

1. Introduction

Microparticle and nanoparticle-based delivery of drugs has been a burgeoning area of interest for well over two decades [1-9]. More recently, the potential for biocompatible, biodegradable polymers to be used in the formation of vaccine vectors has attracted attention in the field of immunology [10-15]. The main impetus behind the development of these particle-based vaccines is to deliver safer and improved vaccines tailored to combat specific diseases through the triggering of appropriate immune effector phenotypes. The enhanced immunostimulatory potential of polymer based particles stems from manifold properties, which include: their capacity to represent a pathogen in terms of size and morphology [16]; the ability to protect their antigenic cargo from rapid degradation and elimination; their propensity to be taken up by dendritic cells; their ability to provide a controlled release of their contents over a sustained period; and their capacity to be co-loaded with antigen(s) and adjuvant(s) of choice thus ensuring co-delivery of antigen and adjuvant to the same dendritic cell [17-24]. Another important feature attributed to particulated vaccine vectors is their enhanced capacity to cross-present antigen compared to antigen in soluble form [25-28].

The manufacture of poly-(diaminosulfide)s (PNSN) in the form of macromolecules has only recently been reported and biodegradable particles made from these polymers were shown to possess low cytotoxicity [29, 30]. PNSN polymers are characterized by having a NSN linkage (R_2NSNR_2) along the backbone and, unlike most polymers, inorganic functional groups are involved in the polymerization reaction (Figure 1). The diaminosulfide functional group degrades at comparable rates to ester bonds at neutral pH, but at much faster rates under acidic conditions [29, 31]. This has the potential advantage of providing triggered or accelerated release of cargo when phagocytosed particles experience lower pH conditions within phagosomal locations of dendritic cells. This difference in degradation kinetics may influence the efficiency of antigen presentation and the phenotype of the immune response.

Vaccines formulated for cancer therapy have been under intense investigation for many decades, however, it has only been relatively recently that promising findings, preclinical and clinical, have given import to this mode of therapy [32, 33]. Many types of vaccine vectors have achieved varying degrees of scrutiny in the tumor immunology field and each have their advantages and disadvantages relating to issues of safety, patient compliance, financial cost, immune potency and labor intensiveness [34, 35]. Delivery of vaccines using attenuated viruses encoding tumor antigen can be a highly efficient way of generating antitumor cellular immune responses, however, such vaccines may be prone to inducing unwanted immune responses or be neutralized by the host's own antibodies [5, 36-39]. Delivery of antigen complexed with alum tends to favor antibody responses at the expense of cellular immune responses and thus is considered unsuitable for use in cancer vaccines [40]. Biodegradable,

biocompatible polymer-based particle vaccines loaded with antigen ($\pm$ adjuvant), of which poly(lactic-co-glycolic acid) (PLGA)-based particles are the most studied [41], are relatively new modes of cancer vaccine delivery vehicles showing promise and garnering increasing interest due to their tunability [42]. However, it is evident that improvements in formulations or alternative polymer materials need to be explored to further enhance the antitumor potential of these microparticle-based vaccines. Hence, the study performed here investigates the potential of antigen/adjuvant-loaded microparticles formed from a novel polymer, PNSN, to induce enhanced antitumor adaptive immunity.

2. Materials and methods

2.1. Materials, mice and cell lines

Sulfur monochloride, dimethylamine, 4,4'-trimethylenedipiperidine, sulfonyl chloride, dichloromethane, rhodamine B, 2-(*N*-morpholino)ethanesulfonic acid (MES) hydrate, albumin from chicken egg with white and Mowiol (poly(vinyl alcohol)) (PVA, Mowiol® 8–88, Mw~67000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylamine is a gas at room temperature, and was therefore condensed inside a graduated cylinder in a -78°C bath before use. Ether, chloroform and methanol were reagent grade and purchased from Fisher Scientific (Pittsburgh, PA). The chemicals, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Thermo Scientific (Rockford, IL, USA). Quant-iT OliGreen® was purchased from Life Technologies (Eugene, OR, USA). Resomer® RG 502 (poly(lactic-co-glycolic acid)) (PLGA) 50:50 with viscosity of 0.2 dL/gm) was purchased from Boehringer Ingelheim KG, Germany. The CpG-containing oligodeoxynucleotide, CpG 1826, which had the following sequence: 5'-T*C*CATGACGTTCTG*A*C*G*T*T-3' (asterisks indicate the presence of phosphorothioate bonds), was purchased from Integrated DNA Technologies (Coralville, IA, USA). Replication-deficient adenovirus type 5 encoding chick ovalbumin (Ad5-Ova), with E1A and E1B genes deleted, was manufactured using standard methods and obtained from the University of Iowa Gene Transfer Vector Core (Iowa City, IA, USA). Ketamine/xylazine mix was purchased from the Office of Animal Resources, University of Iowa (Iowa City, IA, USA). Deionized distilled water was produced by Barnstead Nanopure Diamond Water purification Systems (Dubuque, IA, USA). Sephadex G-25M column (PD-10 column) was purchased from GE Healthcare UK Limited, UK. Genduran silica gel 60 (230–400 mesh) and Basic Alumina Brockman Activity I (60–325 mesh) were purchased from Fisher Scientific and were used for column chromatography during synthesis and characterization of poly(4,4'-trimethylenedipiperdyl sulfide) (PNSN).

E.G7 (thymoma cell line transfected with chick ovalbumin) was obtained from the

American Type Culture Collection (ATCC®, Manassas, VA) and were used at passage numbers 7-12 [43]. The cells were grown in RPMI-1640 (GIBCO, Invitrogen, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA), 1 mM sodium pyruvate (GIBCO, Invitrogen, CA), 10 mM HEPES (GIBCO, Invitrogen, CA), 0.05 mM 2-mercaptoethanol, and 50 mg/ml gentamicin sulfate (Mediatech, Inc., VA) and selection was maintained with 0.4 mg/mL G418 (GIBCO, Invitrogen, CA) in a humidified incubator (Sanyo scientific autoflow, IR direct heat CO₂ incubator) at 37°C containing 95% air and 5% CO₂.

C57BL/6 (H2^b) male mice at the age of 6-8 weeks were obtained from Jackson Laboratories (Bar Harbor, ME) and were used in vaccination studies at age 7-9 weeks. All of the mice were maintained in filtered cages. All animal experiments were carried out in accordance with guidelines and regulations approved by the University of Iowa Institutional Animal Care and Use Committee.

2.2. Synthesis of poly(4,4'-trimethylenedipiperdyl sulfide)

Synthesis of poly(4,4'-trimethylenedipiperdyl sulfide) (PNSN) was carried out by a method described previously [29]. Briefly, dimethylamine (8.01 g) in anhydrous ether (400 mL) was first cooled to -78°C for 44 min. Then 6.00 g of sulfur monochloride was added dropwise to the solution. The solution was stirred for 30 min at -78°C and another 30 min at room temperature. The mixture was then washed with a saturated NaCl aqueous solution. The organic layer was separated, dried over anhydrous magnesium sulfate, and then evaporated to obtain a colorless oil (*N,N'*-dithiobisdipiperdylamine, 6.66 g, 99% yield). Secondly, 50 mL of *N,N'*-dithiobisdipiperdylamine (6.03 g) in anhydrous diethylether (Et₂O) solution was cooled to 0°C for 1 h under nitrogen. Then 5.88 g of sulfonyl chloride was added dropwise to the solution under nitrogen. Then the mixture was stirred for 36 min at 0°C and another 50 min at room temperature to generate *N*-dimethylsulfonyl chloride (8.84 g). Thirdly, 50 mL of *N*-dimethylsulfonyl chloride (8.84 g) in anhydrous Et₂O solution was slowly added to 75 mL of dimethylamine (17.9 g) in anhydrous Et₂O solution at -5°C under nitrogen and stirred for 1.2 h. This mixture was washed with saturated NaCl aqueous solution. The organic phase was separated, dried over anhydrous magnesium sulfate, and then the solvent was removed after freezing the product at -5°C to yield a yellow-green oil (7.0 g). Further purification was achieved by distillation under vacuum at 30°C to obtain a colorless oil (bis(*N,N'*-dimethyl) sulfide, 4.39 g, 46% yield). Finally, 3.71 g of bis(*N,N'*-dimethyl) sulfide was reacted with 6.52 g of 4,4'-trimethylenedipiperidine in 10 mL of chloroform at 60°C for 72 h. After the solvent had evaporated, it was redissolved in 100 mL of dichloromethane. Then PNSN was precipitated into 200 mL of methanol to yield a white-yellow powder (5.61 g, 76% yield). The structure of PNSN was characterized by ¹H and ¹³C NMR spectra obtained from a Bruker DPX 300 at 300 and 75 MHz, respectively. The molecular weight of PNSN was determined by size-exclusion chromatography (SEC; Shimadzu SCL-10A system, Japan using PLgel 5 μm

MIXED-D Varian column).

2.3. Preparation of rhodamine B-conjugated ovalbumin (OVA)

Since PNSN interfered with protein quantitation assays it was necessary to conjugate rhodamine B to OVA so as to determine loading efficiencies. In all experiments performed in this study rhodamine B-conjugated chick egg ovalbumin (OVA) was used in order to facilitate the quantification of OVA using fluorometric spectroscopy. Briefly, to prepare rhodamine B-conjugated OVA, 3.2 mg of Rd, 27.8 mg of EDC and 28.5 mg of sulfo-NHS were dissolved in 3.5 mL, 1.75 mL and 1.75 mL of 0.1 M MES buffer, respectively, and then the three solutions were mixed. The entire volume of this solution was then added to a 35 mL solution of chick egg ovalbumin (5.07 mg/mL) in 0.1 M MES buffer, and mixed. This mixture was stirred with a magnetic stirrer for 2 hour at room temperature concealed from light. Rhodamine B-conjugated OVA was separated by a Sephadex® G-25M column and was freeze-dried for 48 hours to yield a powder form (126.5mg) using a lyophilizer (FreeZone 4.5, Labconco Corporation, Kansas City, Mo, USA). A standard curve of rhodamine B-OVA concentration versus rhodamine B fluorescence (Ex 540 nm/Em 625 nm) was created and loading was calculated through the use of the standard curve. In these studies all references to rhodamine B-conjugated OVA will be abbreviated to "OVA".

2.4. Fabrication and characterization of PNSN microparticles co-loaded with OVA and CpG

PNSN microparticles were fabricated by a double emulsion solvent evaporation method that we have described previously [44]. Briefly, OVA (which had been rhodamine B-labeled (see method section 2.3)) and CpG 1826 were dissolved in 100 μ L of 1% PVA solution as the internal water phase (W_1). Two hundred mg of PNSN was dissolved in 1.5 mL of dichloromethane to form the oil phase (O). W_1 was added to O under microtip probe sonication for 30 seconds on 40% amplitude using a sonicator (Model FB120, Fisher Scientific) to make the primary emulsion (W_1/O). W_1/O was then immediately added to 8 mL of 1% PVA solution (external water phase, W_2) and sonicated for a further 30 seconds on 40% amplitude to generate the secondary emulsion ($W_1/O/W_2$). $W_1/O/W_2$ emulsion was then poured into 22 mL of 1% PVA solution, and magnetically stirred for 2 hours at room temperature in a fume hood to evaporate the dichloromethane. Microparticles were collected by centrifugation at 10,000 $\times g$ for 15 minutes (Accu Spin 400, Fisher Scientific). The microparticles were washed twice with distilled water, and the pellet was frozen overnight at -20°C and then freeze-dried for 40 hours using a lyophilizer (FreeZone 4.5, Labconco Corporation) at -52°C and 0.05 mbar pressure. The microparticles were stored in sealed containers at -20°C. Blank PNSN microparticles were fabricated using the same process without OVA and CpG 1826 in the internal water phase.

Particles size distribution and zeta potential of the microparticles were measured using a

Zetasizer Nano-ZS (Malvern Instrument, Malvern, UK). Briefly, an aliquot of the microparticles was dispersed in distilled water at a concentration of approximately 1 mg microparticles per mL. Particle size distribution was measured by dynamic light scattering with a He-Ne laser at a wavelength of 633 nm, 173° backscatter through a 10 mm cell. Zeta potential was measured by the electrophoretic light scattering method using a He-Ne laser at a wavelength of 633 nm through a folded capillary cell. All measurements were performed at 25°C.

The surface morphology and shape of the microparticles were examined using scanning electron microscopy (SEM). Briefly, the particles were mounted on silicon wafers which were adhered to a SEM stub. The mount was then coated with gold-palladium by an argon beam K550 sputter coater (Emitech Ltd., Kent, England). Images were taken using a Hitachi S-4800 scanning electron microscope (Hitachi High-Technologies, Ontario, Canada), operated at 5 kV accelerating voltage.

The entrapment efficiency (EE) and drug loading (DL) of OVA were determined by a direct method. Briefly, 10 mg of microparticles were dissolved in 4 mL of trifluoroacetic acid:dimethyl sulfoxide (1:9) under sonication (Branson 5200, Branson Ultrasonics Corporation, Danbury, CT, USA) for 30 minutes at 30°C. The absorption of the solution was detected using a fluorescence spectrometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) at Ex: 540 nm/Em: 625 nm. The concentration was calculated from a standard curve. DL and EE were calculated according to Eqs. (1) and (2), respectively.

$$DL (\mu\text{g}/\text{mg}) = \frac{C \times V}{W_p} \quad (1)$$

$$EE (\%) = \frac{W_p \times DL}{W_D} \times 100 \quad (2)$$

Here, C is the concentration of drug in solution ($\mu\text{g}/\text{mL}$), V is the volume of solution (mL), W_p is the weight of microparticles (mg), W_D is the weight of initial drug (mg). DL is expressed by μg of drug encapsulated per mg of microparticles ($\mu\text{g}/\text{mg}$).

EE and DL of CpG in PNSN microparticles were determined by an extraction method. Briefly, 10 mg of microparticles were weighed and placed in a 10 mL vial, then 1 mL of dichloromethane was added to dissolve PNSN. After 3 mL of pH 7.4 phosphate buffer solution (PBS) was added, the vial was sealed and kept rotating by a tube rotator (Scientific Equipment Products Co., Baltimore, MD, USA). At intervals, samples were taken from the aqueous phase for determination of CpG concentration and replenished with an equal

volume of PBS. The concentration of CpG in samples was determined using a fluorescence spectrometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) at excitation wavelength 480 nm and emission wavelength 520 nm after reaction with Quant-iT OliGreen®. Then the concentration was calculated from a standard curve. Drug loading of CpG in PNSN microparticles was calculated according to Eqs. (3). Entrapment efficiency was calculated according to Eq. (2).

$$DL (\mu\text{g}/\text{mg}) = \frac{\sum_{i=1}^n C_i \times V_i}{W_p} \quad (3)$$

Here, n is the total number of samples collected, C_i is the concentration of CpG in aqueous phase ($\mu\text{g}/\text{mL}$) that was collected at i^{th} time, V_i is the volume of aqueous phase (mL) collected at i^{th} time, W_p is the weight of microparticles (mg).

2.5. Prophylactic murine tumor model

Seven to nine week-old male C57BL/6 mice (5 mice per group) were anesthetized by intraperitoneal injection of 100 μL of ketamine/xylazine mixture (contained 17.5 mg of ketamine and 2.5 mg of xylazine per mL of saline), and then injected subcutaneously (on days 0 and 7 (prime/boost) unless otherwise indicated) in the dorsal left flank with the following treatments: (i) naïve (untreated); (ii) blank PNSN microparticles (PNSN(Empty)) at equivalent dose to the OVA and CpG loaded PNSN microparticles; (iii) OVA loaded PNSN microparticles (PNSN(OVA)); (iv) OVA and CpG loaded PNSN microparticles (PNSN(OVA + CpG)); (v) OVA loaded PNSN microparticles plus CpG solution (PNSN(OVA) + CpG sol); (vi) OVA and CpG in solution (OVA sol + CpG sol); (vii) OVA and CpG loaded PLGA microparticles (PLGA(OVA + CpG)); (viii) OVA solution plus incomplete Freund's adjuvant (IFA(OVA)); (ix) adenovirus encoding OVA (Ad5-OVA) (received only a prime (day 0)). For mice treated with microparticles, the microparticles were dispersed in PBS, pH 7.4, immediately prior to injection. For mice treated with CpG solution, the OVA loaded PNSN microparticles or OVA solution was mixed with CpG solution immediately prior to injection. For each group, aside from the Ad5-OVA group, each mouse was treated with the same dose equivalent to 100 μg OVA +/- 13 μg of CpG (except for PLGA microparticles, which was equivalent to 18 μg of CpG). Ad5-OVA treated mice were vaccinated subcutaneously (on day 0 only) with 10^8 pfu ($2.7 \times$

10^9 virus particles). On day 14 and day 21, blood was collected by submandibular bleeds for the determination of OVA-specific CD3⁺ CD8⁺ T lymphocyte levels (see Section 2.6). On day 28 and day 35, serum was collected by submandibular bleeds for the determination of OVA-specific IgG_{2c} and IgG₁ antibody titers (see Section 2.7). On day 35, mice were injected subcutaneously with 1×10^6 OVA expressing E.G7 cells in 100 μ L of PBS into dorsal right flank. Tumor outgrowth was determined by tumor size as a function of time. The diameters and heights of tumors were measured two to three times per week. The tumor volumes were calculated according to Eq. (4) as previously described [45].

$$\text{Tumor volume} = \frac{\pi}{6} \times D_1 \times D_2 \times H \quad (4)$$

Here, D_1 was diameter 1 of the tumor (mm), D_2 was diameter 2 of the tumor (mm), H was the height of tumor (mm).

2.6. Tetramer staining of peripheral blood

Approximately 150-200 μ L of blood was collected by submandibular bleeding and mixed well with ACK buffer ($\text{NH}_4\text{Cl}/\text{KHCO}_3/\text{EDTA}$ solution) to lyse red blood cells. After incubation at room temperature for at least 5 min, cells were washed twice using growth media. The cells were resuspended at $< 10^7/\text{ml}$ and the frequency of OVA-specific CD8⁺ T lymphocytes was determined by tetramer staining, as previously described [46]. The tetramer used was the H-2Kb SIINFEKL Class I iTA_gTM MHC tetramer (Kb-OVA257) labeled with PE (MBLI, Woburn, MA) used at final dilution of 1/100. Surface CD8 and CD3 were stained with anti-CD8-FITC and anti-CD3-PE-Cy5 mAbs (eBioscience, San Diego, CA) respectively. The levels of OVA-specific CD8⁺ T lymphocytes were expressed as a percentage of total CD3⁺ CD8⁺ T lymphocytes in the peripheral blood.

2.7. Determination of anti-OVA antibody levels in sera from peripheral blood

Approximately 250 μ L of blood was collected by submandibular bleeding into an eppendorf microfuge tube. After incubation at room temperature for 1 h, the clot was removed using clean tweezers and the sample was centrifuged at 4°C for 10 min, $3,000 \times g$. The serum was harvested into eppendorf microfuge tubes and stored at -80°C until ready to assay. Serial

dilutions (in PBS + 0.1% tween-20 (Sigma)) of the serum samples were then incubated overnight at room temperature in 96-well Immulon™ 2 HB high binding plates (Thermo Scientific, Waltham, MA, USA) that had been previously coated with 5 µg/mL of OVA solution in PBS. Plates were then washed with 0.1% tween-20 in PBS, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG₁ or IgG_{2c} antibodies (Southern Biotech, Birmingham, AL, USA). Excess antibody was then washed away followed by the addition of p-nitrophenylphosphate (SigmaFAST™, Sigma) in the dark. Absorbance (405 nm) was measured after 15 – 45 min using a SpectraMax® Plus384 microplate reader.

2.8. Statistical analysis

All statistical differences were analyzed by one-way ANOVA analysis of variance followed by a Turkey post-test to compare all pairs of treatment. Survival curves were analyzed using adjustments for multiple comparisons for Log-rank (Mantel-Cox) test using Dunnett's method. All statistical tests were performed using GraphPad Prism software (Prism 5, Version 5.02, La Jolla, CA).

3. Results and Discussion

3.1. Characterization of PNSN

The structure of PNSN was characterized by ¹H NMR and ¹³C NMR (CDCl₃) spectroscopy. The NMR spectra were as follows. ¹H NMR (CDCl₃): δ 1.22(m, 12H), 1.59 (m, 4H), 3.08 (t, 4H, J=11.0 Hz), 3.44 (m, 4H). ¹³C NMR (CDCl₃): δ 23.68, 34.02, 34.96, 36.72, 58.57. It showed that PNSN was synthesized successfully. SEC analysis showed that the number-average molecular weight of PNSN was 7,200 g/mol with a polydispersity index of 3.3.

3.2. Fabrication and characterization of microparticles

PNSN microparticles were fabricated by a double emulsion solvent evaporation method. Figure 2 shows SEM images of PNSN(OVA), PNSN(OVA + CpG), and PNSN(Empty) microparticles. All microparticles were spherical with smooth and nonporous surfaces and ranged in mean size of 1.59 – 1.65 µm diameter. The zeta potential of PNSN(Empty) microparticles was - 24.4 mV and only marginally increased upon encapsulation of OVA or OVA plus CpG into microparticles (Table 1). The entrapment efficiency of OVA into PNSN(OVA) was 37.8%, corresponding to 9.42 µg of OVA encapsulated per mg of microparticles. The entrapment efficiency of OVA and CpG in PNSN(OVA and CpG) was 33.4% and 6.4%,

respectively, corresponding to 7.32 µg of OVA and 0.95 µg of CpG encapsulated per mg of microparticles (Table 1).

Table 1: Particle size, zeta potential, drug loading and entrapment efficiency of PNSN microparticles

Sample (microparticles)	Hydrodynamic diameter (d, µm)		Zeta potential (mV)	Drug loading (µg per mg of microparticles)		Entrapment efficiency (%)	
	Average	PDI		OVA	CpG	OVA	CpG
PNSN(Empty)	1.59	0.45	-24.4±1.7	-	-	-	-
PNSN(OVA)	1.65	0.43	-17.6±0.5	9.42	-	37.8	-
PNSN(OVA + CpG)	1.64	0.62	-23.1±1.3	7.32	0.95	33.4	6.4
PLGA(OVA + CpG)	1.86	0.39	-30.9±0.8	6.83	1.22	34.3	23.0

3.3. Immunogenicity of PNSN microparticle formulations: cell mediated and humoral immune responses

Cell mediated immune responses

Cell mediate immune responses were determined by measuring the levels of OVA-specific CD8+ T lymphocytes. The antigen specificity of the T lymphocytes was determined by staining with a fluorescently tagged tetramer capable of binding to T cell receptors that recognize MHC class I antigen in association with an immunodominant epitope of OVA, SIINFEKL. The T lymphocytes were also co-stained for CD8 to indicate their potential for cytotoxic function, and CD3 to denote that they are T lymphocytes. Mice were vaccinated using a prime/boost regimen described in the methods section using the indicated formulations. Ad5-OVA (prime only) was included as a positive control to verify that the cell

mediated immune response assays were working because of the large detectable responses we have observed in previous studies [45]. All mice were assayed for levels of OVA-specific CD8⁺ T lymphocytes (expressed as a percentage of CD3⁺ CD8⁺ T lymphocytes) in peripheral blood on days 14 and 21 post-prime as described in the methods section (Figure 3A and 3B). On day 14, PNSN(OVA + CpG) vaccinated mice had enhanced OVA-specific CD8⁺ T lymphocyte levels compared to all groups and statistically significant differences were seen when compared to mice vaccinated with PNSN(Empty), PNSN(OVA), as well as naïve mice. The only other vaccination formulations to generate detectable increases in OVA-specific CD8⁺ T lymphocyte responses compared to unvaccinated (naïve) mice were PLGA(OVA + CpG) and OVA sol + CpG sol, however, these increases were not statistically significant. When Ad5-OVA was included in the statistical analysis, it was only Ad5-OVA treated mice that had significant differences (***) $p < 0.001$ compared to all other treatment groups. This was due to the much higher OVA-specific CD8⁺ T lymphocyte responses generated by Ad5-OVA, which were expected. By day 21 most mice from all treatment groups, aside from those treated with Ad5-OVA, had barely detectable levels of OVA-specific CD8⁺ T lymphocytes. The finding that PNSN(OVA + CpG) generated greater cell-mediated immune responses than all other polymer based formulations suggests that these particles may have the capacity to enhance immune based protection against tumors in an antigen-specific manner. This possibility is investigated in section 3.4. These results also emphasize the importance of co-encapsulation of antigen with adjuvant as has been shown with PLGA microparticles previously [20, 21, 47].

Humoral immune responses

Levels of OVA-specific IgG₁ and IgG_{2c} antibodies in the sera of vaccinated mice sampled on days 28 and 35 post-prime were determined using ELISAs (Figure 4). It can be seen that mice vaccinated with PNSN(OVA + CpG) or PLGA(OVA + CpG) generated significantly higher antigen-specific IgG₁ immune responses compared to other treatment groups on both days. In contrast, antigen-specific IgG_{2c} responses were relatively low for PNSN(OVA + CpG) vaccinated mice, displaying IgG_{2c}:IgG₁ ratios of 1:19 and 1:358 on day 28 and day 35, respectively. In comparison, IgG_{2c}:IgG₁ ratios for mice vaccinated with PLGA(OVA + CpG) had higher levels of antigen-specific IgG_{2c} antibody and displayed IgG_{2c}:IgG₁ ratios of 1:1.5 and 1:7 on day 28 and day 35, respectively. All other treatments induced only low to negligible

levels of OVA-specific IgG_{2c} antibodies. Classical immunological dogma would therefore credit the PLGA-based vaccine to have incited a more Th1-biased immune response than the co-loaded PNSN formulation [48]. However, results from section 3.2 (cell-mediated immunity) and section 3.4 (tumor protection and survival studies) are inconclusive and are discussed further in section 3.4.

3.4. Tumor protection and survival studies

Mice were challenged subcutaneously with 1×10^6 E.G7 tumor cells 4 weeks after the booster vaccination and were monitored for tumor volume (Figure 5) and survival (Figure 6). Statistical analyses of the data were performed in three ways: 1) assessing immunoprotective potency of each vaccine by comparing tumor volumes on day 9 post-tumor challenge (Figure 5B) and determining statistically significant differences (Table 2), 2) determining statistically significant differences in tumor volumes on day 14 post-tumor challenge (day 14 being last day where no mice had been sacrificed due to tumor size) (Figure 5C & Table 2) and, 3) comparing survival curves (Figure 6). Day 9 was chosen as the time point to compare each group for the immunoprotective potency engendered by each vaccine since this is enough time to ensure the appearance of measurable tumors in unprotected mice [49]. Results show that most of the formulations containing OVA, aside from PNSN(OVA) and PNSN(OVA) + CpG sol, afforded mice with a significant degree of tumor protection (Table 2). Thus it would appear that co-loading CpG and OVA into PNSN microparticles was of benefit in terms of tumor protection. The importance of co-encapsulation of antigen and adjuvant has been shown previously using PLGA microparticles [47]. It is likely that most of the protective vaccine formulations mediated protection through the generation of OVA-specific CD8⁺ T lymphocytes despite only modest increases in the levels of these cells being observed (see Figure 3A).

The most enigmatic result from the day 9 tumor volume measurements (Figure 5C) was that achieved with IFA(OVA) where significant protection occurred compared to unvaccinated mice and mice vaccinated with PNSN(Empty) or PNSN(OVA), however, no cell-mediated response was detected (see Figure 3). By day 14 post-tumor challenge two of the five naïve mice were exhibiting tumor regression which would explain why none of the vaccinations containing OVA were significantly different to the naïve group with respect to tumor volume

on day 14 (Figure 5C and Table 2). The PNSN(Empty) treated group displayed a significantly enhanced mean tumor volume on day 14, but not on day 9, compared to the naïve group, possibly due to the fact that none of the mice in the PNSN(Empty) vaccinated group had tumors that underwent spontaneous regressions (see Figure 5A). All vaccination groups involving OVA possessed significantly lower mean tumor volumes (on day 14) than the PNSN(Empty) treated group and, in most cases, this is likely to be due to the immunoprotective responses induced by each formulation which were already evident by day 9. However, this explanation may not so readily apply for the mice vaccinated with PNSN(OVA) alone or PNSN(OVA) + CpG sol since they exhibited only marginal and non-significant tumor protection by day 9 (Figure 5B) and at least some of these mice may have had tumors that underwent spontaneous regressions as suggested by the growth kinetics shown in Figure 5A. The finding that OVA sol + CpG sol was significantly immunoprotective compared to PNSN(Empty) treated mice is probably an indication of the immunogenic nature of the tumor model used here. E.G7 tumor cells express OVA, a xenogeneic protein, and are considered sensitive to immune-mediated rejection [43, 50]. Thus formulations that cannot perform at least as well as OVA sol + CpG sol in terms of tumor protection and overall survival are not likely to perform well in more rigorous circumstances, such as in a therapeutic setting or against a less immunogenic tumor type.

When the survival curves were analyzed comparing all treatment groups to the PNSN(Empty) group the only groups to be significantly different to the PNSN(Empty) treated mice were the mice vaccinated with Ad5-OVA (*P = 0.032) and PNSN(OVA + CpG) (**P = 0.0008) (Figures 6A & 6B). These survival data and the percentage of mice tumor-free (Table 3) at the termination of the experiment (day 60) highlight the enhanced antitumor effect of the PNSN(OVA + CpG) formulation over other formulations. That PNSN(OVA + CpG) vaccinated mice showed a trend toward greater survival over an adenovirus-based vaccine and also PLGA(OVA + CpG) is highly encouraging and significant.

Thus PNSN(OVA + CpG) compared favorably with PLGA(OVA + CpG), the latter of which has been more intensively studied and shown promising preclinical results with respect to the generation of cell-mediated responses and the induction of antitumor immunity [47].

Table 2: Significant differences in tumor volume on days 9 and 14 post tumor challenge.

<u>Day 9</u>	Naïve	PNSN (Empty)	OVA sol + CpG sol	PNSN(OV A)	PNSN (OVA) + CpG sol	PNSN (OVA + CpG)	PLGA(OVA + CpG)	IFA(OVA)	Ad5-OVA
naïve			**			**	**	*	**
PNSN (Empty)			**			**	**	**	***
PNSN (OVA)			*			*	*		*
<u>Day 14</u>	Naïve	PNSN (Empty)	OVA sol + CpG sol	PNSN(OV A)	PNSN (OVA) + CpG sol	PNSN (OVA + CpG)	PLGA(OVA + CpG)	IFA(OVA)	Ad5-OVA
naïve		**							
PNSN (Empty)	**		***	***	***	***	***	***	***

Table 3: Percentage of mice tumor-free on days 9, 14 and 60 post tumor challenge.

Percentage of mice tumor-free									
	Naïve	PNSN(Empty)	OVA sol + CpG sol	PNSN(Ova)	PNSN(Ova) + CpG sol	PNSN(Ova + CpG)	PLGA(Ova + CpG)	IFA(Ova)	Ad5-Ova
Day 9	0	0	80	0	0	100	50	75	100
Day 14	0	0	40	0	20	100	75	100	100
Day 60	20	0	20	0	40	75	25	0	40

Conclusion

The key findings of this study were that a new biodegradable polymer, PNSN, could be used to form particles capable of being co-loaded with antigen (OVA) and adjuvant (CpG), which upon vaccination, displayed the capacity to generate detectable cell-mediated immune responses, as defined by increased levels of OVA-specific CD8+ lymphocytes, and promoted increased survival of tumor challenged mice. In this prophylactic setting the antitumor effect of these co-loaded microparticles was at least comparable with that achieved with co-loaded

PLGA microparticles or Ad5-OVA, suggesting that vaccine delivery systems fabricated from polydiaminosulfides may have promise in cancer therapy.

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Figure legends

Figure 1: Chemical structure of poly(4,4'-trimethylenedipiperdyl sulfide (PNSN)

Figure 2: SEM microphotographs of (A) PNSN(Empty) microparticles, (B) PNSN(OVA) microparticles, (C) PNSN(OVA + CpG) microparticles. Scale bar (white) = 5 μ m.

Figure 3: OVA-specific CD8⁺ T lymphocyte levels. Peripheral blood lymphocytes isolated from all mice from all indicated treatment groups were co-stained for the presence of Ova-specific T cell receptors, CD3, and CD8 on days 14 (A) and 21 (B) post prime as described in methods section. Statistical analysis was performed excluding the Ad5-OVA treated group (Insets). One-way ANOVA with Tukey's post-test was performed. Data are presented as mean $\pm$ SEM. (*p < 0.05).

Figure 4: Serum titers of OVA-specific IgG₁ and IgG_{2c} OVA-specific antibodies after vaccination with indicated formulations. Mice were prime/boosted on days 0/7 and then on day 28 and day 35 sera were collected and assayed for OVA-specific IgG₁ or IgG_{2c} antibody using ELISA as described in the methods section. Titers for OVA-specific IgG₁ on day 28 (A) and day 35 (B); titers for OVA-specific IgG_{2c} on day 28 (C) and day 35 (D). Statistical significances were determined using one-way ANOVA followed by Tukey's post-test. Error bars represent SEM. For (A) ***¹ indicates that PNSN(OVA + CpG) is significantly different (P < 0.001) to all other treatments other than PLA(OVA + CpG) and ***² indicates that PLGA(OVA + CpG) was significantly different (P < 0.001) to all other treatments other than PNSN(OVA + CpG). For (B) ***³ indicates that PNSN(OVA + CpG) was significantly different (P < 0.001) to all other treatments other than PLGA(OVA + CpG) and IFA(OVA), *¹ indicates PNSN(OVA + CpG) is significantly different (P < 0.05) to IFA(OVA) and ***⁴ indicates PLGA(OVA + CpG) is significantly different (P<0.001) to all groups except PNSN(OVA + CpG). For (C) *² indicates that PLGA(OVA + CpG) is significantly different (P < 0.05) to all other treatments. For (D) *³ indicates that PLGA(OVA + CpG) is significantly different to all other treatments except IFA(OVA).

Figure 5: The prophylactic antitumor effect of vaccinating mice with different OVA and CpG formulations. Thirty-five days prior to subcutaneous challenge with E.G7 tumor cells, mice (5 mice/group) were vaccinated with the indicated formulations on day 0 (prime) and day 7 (boost)*¹. (A) Tumor volumes were recorded and each curve represents the tumor growth for each individual mouse. Tumor volumes from each treatment group were compared statistically on day 9 (B) and day 14 (C) using one way ANOVA followed by Tukey's post-test. Ψ^1 indicates statistical differences were observed and are summarized in Table 2.

*¹ Ad5-OVA vaccinated mice did not receive a boost.

Figure 6: Survival curve of mice bearing E.G7 tumors. Thirty-five days prior to subcutaneous challenge with E.G7 tumor cells, mice (5 mice/group*¹) were vaccinated with the indicated formulations on day 0 (prime) and day 7 (boost)*². Survival curves for each treatment group

are displayed in (A and B) Survival curves were analyzed using adjustments for multiple comparisons (of all groups versus PNSN(Empty)) for the Log-rank test with Dunnett's method and significant differences between them displayed. * = $P < 0.05$; *** = $P < 0.001$.

*¹ Three treatment groups had 1 mouse removed from the survival study due to deaths unrelated to tumor growth. Two mice were ethically sacrificed due to self-harm (one from PNSN(OVA+CpG) group and one from PLGA(OVA+CpG) group. One mouse died subsequent to anesthesia administration (IFA(OVA)).

*² Ad5-OVA vaccinated mice did not receive a boost.

Table 1: Hydrodynamic diameter, zeta potential, drug loading and entrapment efficiency of PNSN microparticles

Table 2: Significant differences in tumor volume on days 9 and 14 post tumor challenge. Companion table to Figure 5 showing significant differences in tumor volumes on day 9 and day 14 post tumor challenge. One-way ANOVA plus Tukey post-test was performed. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Table 3: Percentage of mice tumor-free on days 9, 14 and 60 post tumor challenge.

FIGURES

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6