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Poly(ester-anhydride):poly(β-amino ester) micro- and nanospheres: DNA encapsulation and cellular transfection

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Abstract

Poly(ester-anhydride) delivery devices allow flexibility regarding carrier dimensions (micro- versus nanospheres), degradation rate (anhydride versus ester hydrolysis), and surface labeling (through the anhydride functional unit), and were therefore tested for DNA encapsulation and transfection of a macrophage P388D1 cell line. Poly(L-lactic acid-*co*-sebacic anhydride) and poly(L-lactic acid-*co*-adipic anhydride) were synthesized through melt condensation, mixed with 25 wt.% poly(β-amino ester), and formulated with plasmid DNA (encoding firefly luciferase) into micro- and nanospheres using a double emulsion/solvent evaporation technique. The micro- and nanospheres were then characterized (size, morphology, zeta potential, DNA release) and assayed for DNA encapsulation and cellular transfection over a range of poly(ester-anhydride) copolymer ratios. Poly(ester-anhydride):poly(β-amino ester) composite microspheres (6–12 μm) and nanospheres (449–1031 nm), generated with copolymers containing between 0 and 25% total polyanhydride content, encapsulated plasmid DNA (≥20% encapsulation efficiency). Within this polyanhydride range, poly(adipic anhydride) copolymers provided DNA encapsulation at an increased anhydride content (10%, microspheres; 10–25%, nanospheres) compared to poly(sebacic anhydride) copolymers (1%, microspheres and nanospheres) with cellular transfection correlating with the observed DNA encapsulation.

Keywords: Poly(ester-anhydride); Polymeric biomaterials; Microspheres; Nanospheres; Gene delivery; Transfection

1. Introduction

Polymeric materials have found wide use in gene delivery applications (Luo and Saltzman, 2000). Examples include poly(α-hydroxy esters) (i.e., poly(D,L-lactic-*co*-glycolic acid) (PLGA)) (Wang et al., 1999), poly(β-amino esters) (Lynn and Langer, 2000), polyanhydrides (Mathiowitz et al., 1997), poly(ethylene imine) (Boussif et al., 1995), and poly(L-lactide-*co*-L-lysine) (Barrera et al., 1993). These polymers generally serve as gene carriers through either electrostatic complexation or physical entrapment for the eventual intracellular delivery of their gene payload.

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One formulation method used to physically entrap DNA involves generating polymeric micro- or nanospheres (Wang et al., 1999; Perez et al., 2001). However, polymeric gene delivery strategies can be limited depending on the sphere size to be tested. Microspheres have great potential to passively target cell types capable of scavenging particles in the range of 1–10 μm (i.e., antigen presenting cells such as macrophages or dendritic cells) (Walker et al., 2001); whereas, somatic cell targeting via systemic administration would most likely require nanospheres (10–1000 nm) (Kreuter, 1992; Desai et al., 1997). The ability to formulate both micro- and nanoscale gene carriers could have significant implications for gene delivery to a wide cellular target base.

Successful gene delivery requires efficient DNA encapsulation during formulation and intracellular gene delivery once the carriers have been endocytosed. To this end, poly(β-amino esters) have been synthesized to enhance intracellular gene delivery (Lynn et al., 2001). Previous gene delivery efforts using microspheres composed of PLGA (75 wt.%) and a poly(β-amino ester) (25 wt.%) have greatly enhanced the cellular transfection capabilities of these carriers (presumably through the pH responsive nature of the poly(β-amino ester) facilitating endosomal escape) (Lynn et al., 2001). This polymer combination provides an advantage over microspheres formulated with PLGA alone (Hedley et al., 1998).

In addition to poly(α-hydroxy esters), polyanhydride micro- and nanospheres have been used for gene delivery applications (Mathiowitz et al., 1997; Sandor et al., 2002). These two polymers differ significantly in rate and mode of degradation; however, both are safe and established biomaterials (Uhrich et al., 1999). Such differences in biodegradation may influence the final outcome of drug or gene controlled delivery. Additionally, the surface labeling properties of polyanhydride micro- and nanospheres offer another way to influence the cellular interactions of these carriers (Gao et al., 1998). Recently, poly(α-hydroxy ester-anhydride) copolymers have been synthesized (Storey and Taylor, 1997; Korhonen and Seppala, 2001; Slivniak and Domb, 2002). These copolymers possess both the degradation and surface labeling properties of individual polyesters and polyanhydrides and may therefore provide extended advantages compared to either polymer alone.

In this work, we present the formulation of micro- and nanoscale gene carriers using poly(ester-anhydride) copolymers and poly(β-amino esters). Such carriers were characterized with regards to sphere size, morphology, surface potential, and DNA release. The spheres were then tested for gene encapsulation and cellular transfection using a macrophage (P388D1) cell line.

2. Materials and methods

2.1. Materials

Sebacic acid (99%, Sigma, St. Louis, MO) was recrystallized twice in ethanol. Adipic acid (>99.5%, Fluka, Buchs, Switzerland) was recrystallized once in ethanol. Poly(L-lactic acid) (2000 Da) and poly(vinyl alcohol) (PVA, Polysciences, Warrington, PA, 88 mol% hydrolyzed, M_w ~25,000) were obtained from Polysciences (Warrington, PA). Acetic anhydride (99.5%), methylene chloride (anhydrous), petroleum ether (anhydrous), and ethyl ether (anhydrous) were used as received from Aldrich; Milwaukee, WI. Poly(vinyl alcohol), ethylenediaminetetraacetic acid (EDTA, 99%, Sigma), lactose (Mallinckrodt, Paris, KY), NaCl (Mallinckrodt), and D(+)-trehalose dihydrate (Sigma) were used during micro- and nanosphere formulations.

2.2. Polymer synthesis and characterization

Sebacic or adipic acid was activated by heating in acetic anhydride (1:10, w/v) for 25 min at 120 °C with stirring (Fig. 1). Excess acetic anhydride was removed under vacuum, and the remaining off-white prepolymer was dissolved in dichloromethane (smallest volume needed to dissolve) and precipitated (1:10, v/v) in a 1:1 ethyl ether:petroleum ether mixture. The prepolymers were filtered, dried under vacuum, and stored at −20 °C.

Poly(L-lactic acid), containing hydroxyl and carboxylic acid end groups, was activated by heating in acetic anhydride (1:10, w/v) for 25 min at 120 °C with stirring (Fig. 1). Excess acetic anhydride was removed under vacuum. The activated prepolymer was then dried under vacuum, stored at −20 °C, and used without further purification.

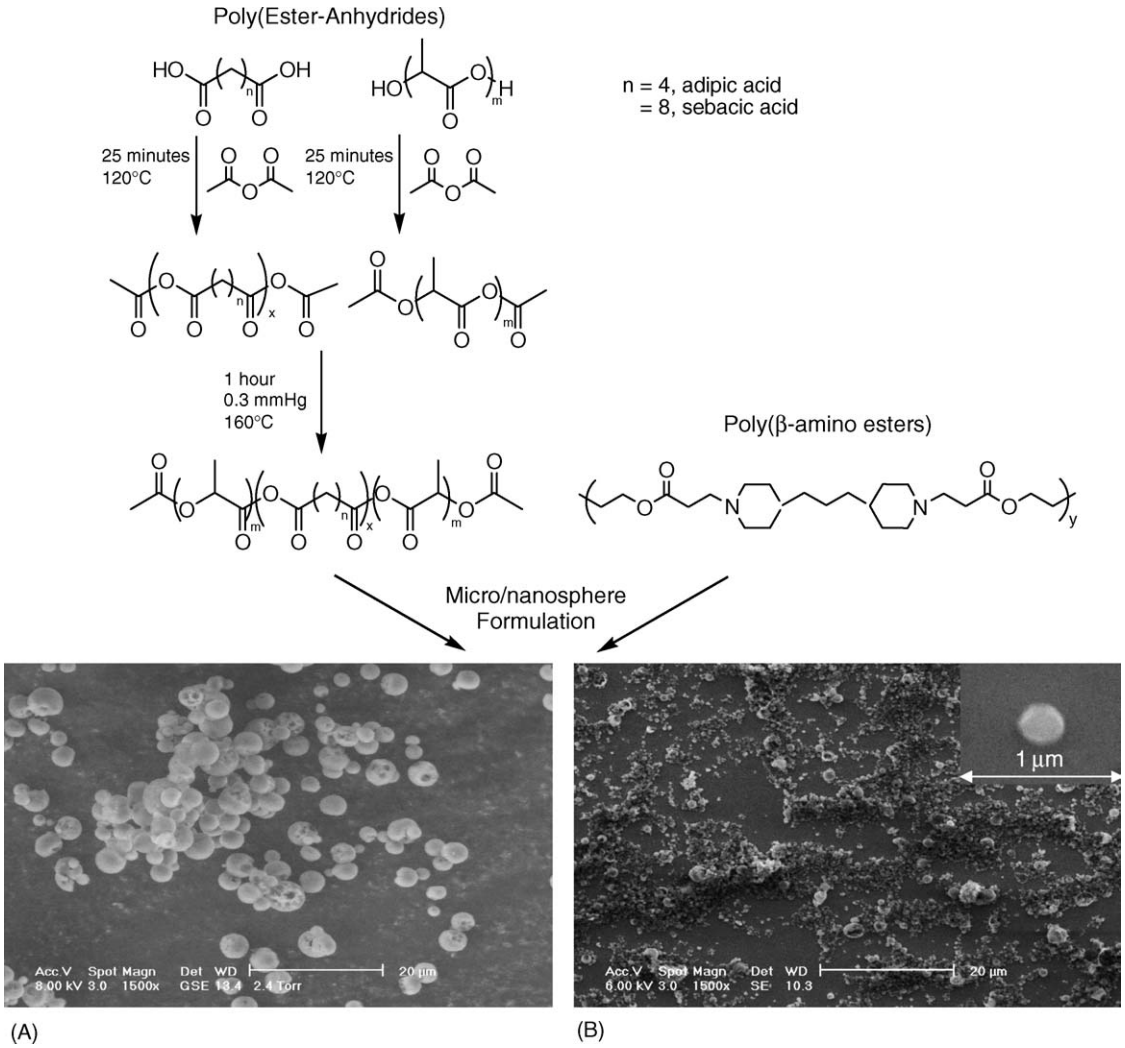

Fig. 1. Synthetic scheme for poly(ester-anhydride) copolymers leading to micro- or nanosphere formation. Both sphere types included 25% (wt.%) poly(β-amino ester). Poly(L-lactic acid-*co*-adipic anhydride):poly(β-amino ester) (1% adipic anhydride) microspheres (A) and nanospheres (B) formulated with plasmid DNA. Scale bar at bottom of both pictures represents 20 μm.

Poly(L-lactic acid-*co*-sebacic anhydride) or poly(L-lactic acid-*co*-adipic anhydride) copolymers were generated according to the scheme presented in Fig. 1. Different mass ratios for the poly(sebacic anhydride) or poly(adipic anhydride) prepolymers and the poly(L-lactic acid) prepolymer were mixed and heated to 160 °C (and allowed to melt) before a vacuum of 0.3 mmHg was applied for 1 h. The copolymers were then stored at −20 °C. The poly(β-amino ester) was synthesized as previously reported (Lynn and Langer, 2000; Lynn et al., 2001).

The pre- and copolymers were characterized by gel permeation chromatography (GPC) and ^{1}H NMR to determine polymer molecular weight and chemical structure, respectively (Table 1). GPC analysis was conducted using a Waters [Milford, MA] 515 HPLC pump, two Styragen HR4 columns [7.8 mm × 300 mm] in series, a 2410 refractive index detector, chloroform (Aldrich) as the solvent system (1 ml/min) and polystyrene MW standards (Polysciences). ^{1}H NMR was conducted using $CDCl_3$ (Aldrich) as the solvent and a Varian Unity 300 spectrometer

Table 1
Polymer and microsphere/nanosphere characterization

Polymer[a] % ratio	M_w[b]	Size[c] microsphere (μm)/nanosphere (nm), standard deviations in parentheses	Zeta potential[d] (mV) microsphere/nanosphere, standard deviations in parentheses
100:0 PLA:PSA	6850	6.8 (3.8)/987.8 (27.3)	63.6 (0.73)/37.7 (1.23)
99:1 PLA:PSA	7840	6.8 (3.8)/1030.8 (23.4)	54.4 (0.15)/36.2 (1.01)
95:5 PLA:PSA	8380	8.9 (4.8)/616.7 (11.9)	45.6 (0.84)/37.0 (0.65)
90:10 PLA:PSA	10900	12.1 (5.3)/580.8 (19.1)	29.4 (1.68)/32.2 (1.63)
75:25 PLA:PSA	13800	8.7 (5.4)/456.9 (15.5)	31.9 (0.86)/32.1 (0.90)
50:50 PLA:PSA	14150	6.6 (2.7)/449.0 (9.6)	26.2 (1.03)/26.8 (0.20)
25:75 PLA:PSA	16700	6.2 (2.6)/537.8 (13.8)	29.2 (0.39)/27.9 (1.57)
0:100 PLA:PSA	13400	6.0 (2.6)/546.8 (20.3)	27.3 (1.82)/29.0 (0.80)
99:1 PLA:PAA	7750	6.7 (3.4)/728.1 (25.9)	50.7 (1.08)/42.3 (0.77)
95:5 PLA:PAA	7920	7.7 (3.6)/841.7 (21.1)	50.2 (1.63)/39.5 (0.90)
90:10 PLA:PAA	7170	8.6 (4.7)/586.2 (25.2)	42.2 (2.23)/37.3 (0.59)
75:25 PLA:PAA	6890	10.7 (5.8)/487.1 (11.5)	19.8 (1.33)/17.0 (0.96)
50:50 PLA:PAA	7670	10.9 (6.7)/637.5 (22.4)	15.5 (0.25)/16.1 (0.36)
25:75 PLA:PAA	9720	10.6 (6.3)/704.3 (29.0)	16.2 (1.12)/−2.8 (0.79)
0:100 PLA:PAA	10100	8.2 (5.7)/455.0 (14.7)	18.4 (0.93)/−6.8 (0.31)

[a] PLA:PSA, poly(L-lactic acid-*co*-sebacic anhydride) copolymer; PLA:PAA, poly(L-lactic acid-*co*-adipic anhydride) copolymer.
[b] M_w calculated by GPC.
[c] Sphere size by Coulter counter analysis (microspheres) and QELS (nanospheres).
[d] Zeta-potential measured using a ZetaPALS light scattering detector.

(300 MHz). As previously observed for this particular synthetic scheme, final copolymer M_w values were between 5 and 20 kDa with an increase in molecular weight observed with increasing polyanhydride content (Slivniak and Domb, 2002).

2.3. Microsphere formulation

Microspheres were generated using a double emulsion/solvent evaporation procedure. Initially, the polymer (150 mg copolymer, 50 mg poly(β-amino ester)) was dissolved in methylene chloride (4 ml). Next, 100 μl of an aqueous 300 mM lactose, 1 mM EDTA solution containing 1 mg plasmid DNA (pCMV-Luc encoding the firefly luciferase gene under a cytomegalovirus promoter and purchased from Elim Biopharmaceuticals, Hayward, CA) was added to the organic polymer solution, and the aqueous and organic phases were emulsified by sonication (Vibra Cell, Sonics & Materials, Inc., Danbury, CT, 50 W power output for 10 s using a stepped microtip probe). The primary emulsion was then transferred to 50 ml of an aqueous 1% PVA, 0.5 M NaCl solution for a 30 s homogenization (L4RT-A, Silverson equipped with a 3/4 in. tubular frame with a square hole high shear screen, 7500 rpm) to form a secondary emulsion. The secondary emulsion was added to an aqueous 100 ml 0.5% PVA (containing 0.5 M NaCl) solution and stirred to evaporate the organic solvent (using a stirplate at room temperature for 2.5 h and an additional 0.5 h at 4 °C [needed to help preserve microsphere morphological integrity]). The microspheres were then washed (by centrifugation [150 × *g*, 10 min] and resuspension) three times with cold double-distilled water (pH 7, with all washes at 4 °C), frozen with liquid nitrogen, lyophilized to a dry powder using a VirTis Freezemobile 25EL (Gardiner, NY) and standard Labconco freeze dry flasks according to the manufacturer's instructions, and stored at −20 °C.

2.4. Nanosphere formulation

Nanospheres were also formulated using a double-emulsion/solvent evaporation technique. Fifty microlitres of the 300 mM lactose, 1 mM EDTA solution containing 0.5 mg plasmid DNA was added to 1.5 ml dichloromethane (containing 75 mg copolymer and 25 mg poly(β-amino ester)). The aqueous and organic phases were emulsified by sonication (75 W, 10 s) and added to a 6 ml 3% PVA, 0.5 M NaCl solution.

After sonication (75 W, 20 s, on ice), this secondary emulsion was added to a 35 ml 0.5% PVA, 0.5 M NaCl solution and stirred for 3 h (2.5 h at room temperature and 0.5 h at 4 °C). The samples were then washed three times by centrifugation (3600 × *g*, 15 min), mixed with at least 1× by weight D(+)-trehalose (if needed; i.e., added to those samples that exhibited aggregation when lyophilized alone), frozen, lyophilized, and stored at −20 °C.

2.5. Micro- and nanosphere characterization

Micro- and nanospheres were characterized for particle size, morphology, zeta potential, and DNA release. For sizing and zeta potential measurements, spheres were resuspended at 0.1 mg/ml in filtered double-distilled water and sonicated (35 W, 5 s). A Coulter microparticle analyzer (Multisizer 3, Beckman Coulter, Miami, FL) was used to generate an average diameter for the microspheres (volume average; sample count: 50,000 microspheres). Nanospheres were sized using quasi-elastic laser light scattering (QELS). A ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY, 15 mW laser, incident beam = 676 nm) was used to measure zeta potentials for both micro- and nanospheres with samples measured at 0.1 mg/L in water. An environmental scanning electron microscope (ESEM, Philips XL30) was used to further confirm sphere size and overall morphology. For microsphere SEM analysis, lyophilized samples were observed directly; whereas, for nanospheres, to liberate the spheres from trehalose and to properly disperse individual spheres, the final lyophilized product was resuspended in double-distilled water (1 mg in 0.5 ml), sonicated (2 s) and washed (via centrifugation, 3×), and 50 μL air dried for analysis. To measure DNA release, 5 mg of poly(L-lactic acid-*co*-adipic anhydride):poly(β-amino ester) micro- or nanospheres were resuspended by sonication (35 W, 2 s) in 0.75 mL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.7, preheated to 37 °C). The suspension was then incubated at 37 °C under rotation. At the indicated time points, the samples were centrifuged and the supernatants stored at −80 °C until analysis; the pelleted spheres were resuspended in fresh (preheated) buffer for further incubation. For analysis, the stored supernatants were measured for DNA content at A_{260} (as described for micro- and nanosphere DNA encapsulation). Samples were measured in triplicate ($n = 3$).

2.6. Micro- and nanosphere DNA encapsulation

DNA encapsulation for both micro- and nanospheres was determined by first resuspending 2.5 mg spheres in 0.25 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.7, Sigma). One milliliter dichloromethane was added and the two phases were then mixed via rotation for 1.5 h at room temperature. After this time, the mixture was centrifuged and the aqueous phase carefully removed and analyzed at A_{260} (using a SPECTRAmax PLUS 384 UV–vis 96 well plate reader (Molecular Devices Corp., Sunnyvale, CA) and flat bottom, acrylic, UV transparent 96 well plates); samples were measured in duplicate and compared to a pCMV-Luc DNA standard curve (0–70 μg/mL in TE buffer). Samples with no DNA were tested to ensure that soluble polymer fragments or residual solvent did not interfere with the UV signal. To obtain % DNA encapsulated, the recovered DNA per mg polymer was compared to the initial DNA per mg polymer added during micro- or nanosphere formation.

2.7. In vitro transfection assay

The macrophage P388D1 cell line was maintained in media prepared as follows: to 500 ml 1640 RPMI Media (Gibco™, Invitrogen Corp., Grand Island, NY), 50 ml fetal bovine serum (certified heat inactivated, Gibco™, Invitrogen Corp.), 5 ml 1 M HEPES buffer solution (Gibco™, Invitrogen Corp.), 5 ml 100 mM MEM sodium pyruvate solution (Gibco™, Invitrogen Corp.), 5 ml penicillin-streptomycin solution (Gibco™, Invitrogen Corp.), and 1.25 g D(+)-glucose (Sigma) were added. The media was then filtered (0.2 μm), stored at 4 °C, and prewarmed to 37 °C before subsequent use. Assay conditions were performed in 96 well plates: cellular protein measurements were performed using tissue culture treated, sterile, polystyrene FALCON Microtest™ 96 plates (Becton Dickinson, Franklin Lakes, NJ); whereas, tissue culture treated, flat bottom, white, polystyrene 96 well plates were used for luciferase quantification. Cultured P388D1 cells were seeded at a density of 30,000 cells/well (in 150 μl media). The cells were allowed to adhere for 24 h at 37 °C and 5% CO_2 in a humidified incubator.

At this point, micro- or nanospheres were resuspended in media and sonicated (35 W, 3 s) before replacing the original media in the 96 well plates with final micro- or nanosphere concentrations of either 10 or 50 μg/ml (added at 100 μl per well). Unencapsulated plasmid DNA was also added to wells at concentrations ranging from 10% to 1000% the theoretical encapsulation efficiency of the microspheres. The plates were then incubated as before for an additional 24, 48, or 72 h (the maximum permissible length with this assay before media nutrients were depleted and cell death occurred). At each time point, the plates were washed twice with 150 μl PBS each time. The remaining cells were then lysed using Glo Lysis Buffer (Promega, Madison WI). To assess cellular protein content, a Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) was used and the plates spectrophotemetrically read at A_{562} according to the assay's instructions. To quantify luciferase production, a Bright Glo™ Luciferase Assay System (Promega) was used (together with a Mithras LB 940 luminescence plate reader, each well read for 2 s (Berthold Technologies, Oakridge, TN)) to measure relative light units produced through the enzymatic action of luciferase; the relative light unit data were then converted to fg luciferase through a standard curve of luciferase amount versus relative light units. Together with the BCA total protein assay, data were presented as fg luciferase/mg total cellular protein. Samples were tested in triplicate ($n = 3$).

Statistical analysis was performed using a Student's *t*-test with a minimum confidence level of 0.05 for statistical significance. This was used to compare transfection levels at the same weight% anhydride levels between poly(L-lactic acid-*co*-sebacic anhydride) or poly(L-lactic acid-*co*-adipic anhydride) derived micro- and nanospheres.

3. Results and discussion

3.1. Micro- and nanosphere characterization

The average sizes for both poly(ester-anhydride):poly(β-amino ester) micro- and nanospheres and the zeta potentials associated with these formulations are shown in Table 1. Nanosphere formulations required the addition of D(+)-trehalose to prevent nanosphere aggregation post lyophilization. Microspheres were in the size range of 6–12 μm; whereas, nanospheres were between 450 and 1000 nm. Each formulation was also confirmed for correct spherical size and morphology using scanning electron microscopy (Fig. 1 and data not shown). Zeta potential measurements generally showed a positive surface potential owing to the inclusion of 25% poly(β-amino ester) (since micro- or nanospheres formed without poly(β-amino ester) demonstrated negative potentials, data not shown). Additionally, micro- and nanosphere zeta potentials tended to decrease with increasing polyanhydride copolymer content.

The flexibility to generate a variety of sphere sizes opens the possibility for multiple administration routes. For some applications, such as subcutaneous, pulmonary, or oral forms of administration, microspheres have been a standard controlled release strategy (Gupta et al., 1990; Mathiowitz and Langer, 1992; Mathiowitz et al., 1997). Alternatively, drug or gene carriers in the nanosphere range allow potential systemic delivery; although, even smaller sphere sizes could aid this effort (Gref et al., 1994). Nanosphere systemic gene delivery would be aided greatly by the ability to modify such carriers to target specific cell types. The anhydride functional unit within these copolymers allows a simple route to surface modification that is possible even at low anhydride levels (Pfeifer et al., 2005).

Differences in degradation rates are expected depending on the poly(ester-anhydride) copolymer ratio. This was particularly evident for nanospheres derived from adipic acid. During light scattering measurements, nanospheres containing a high poly(adipic anhydride) copolymer ratio showed a tendency to increase in diameter over time, possibly indicating swelling due to polymer hydrolysis and increased water uptake by the spheres (with aggregation being another possibility). In addition, zeta potential values for nanospheres containing the highest poly(adipic anhydride) ratios reverted from positive to negative values, potentially indicating a rapid anhydride degradation pattern which produces more free (negatively charged) carboxylic acid groups and leads to a more negative overall zeta potential. Aqueous nanosphere suspensions also showed a more pronounced initial pH decrease with poly(adipic anhydride) copolymers as compared to poly(sebacic anhydride) copolymers. Finally, the in vitro DNA release rate from adipic acid derived micro- and nanospheres increased with adipic

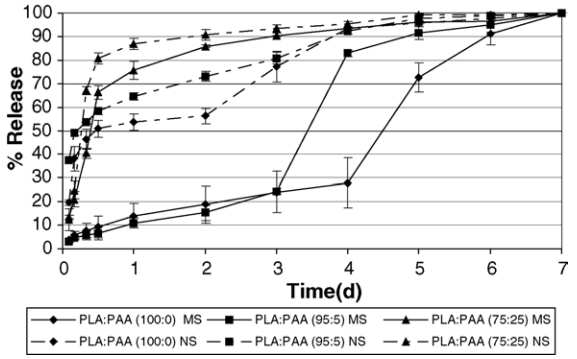

Fig. 2. DNA release from microspheres (MS, solid lines) and nanospheres (NS, dashed lines) containing 0, 5, and 25% poly(adipic anhydride) content. Data presented as percent total DNA released. PLA:PAA, poly(L-lactic acid-*co*-adipic anhydride) copolymer.

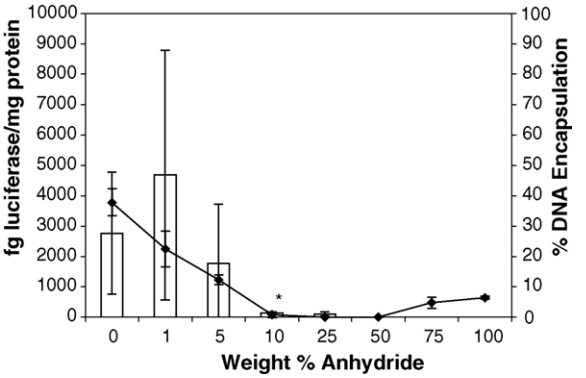

Fig. 3. Transfection (bars; left axis; 24 h time point) and gene encapsulation (data points; right axis) data for poly(L-lactic acid-*co*-sebacic anhydride):poly(β-amino ester) microspheres. The asterisk (*) denotes statistical significance ($p < 0.05$) between the measured transfection levels at the same weight% anhydride content compared to Fig. 5.

anhydride content (Fig. 2). The plot reveals a faster release from nanospheres (with a more pronounced burst effect) compared with microspheres. Sebacic acid derived nanospheres showed a similar DNA release trend but over a longer time period (data not shown). All of these observations support a faster degradation rate for adipic acid based polymers (as opposed to sebacic acid) due to the less hydrophobic nature of this starting unit; this property (in addition to gene encapsulation) may also help explain differences in degree of cellular transfection (see below) between copolymers containing the same polyanhydride content (i.e., 5% sebacic anhydride versus 5% adipic anhydride). Likewise, the differences in DNA release rate presented in Fig. 2 illustrate how anhydride content affects both micro- and nanosphere release profiles, and though not thoroughly explored in this study, these differences could correlate to DNA transfection. Though these degradation rates may have both advantages and disadvantages, freedom to influence gene carrier degradation through both synthetic and formulation routes is viewed as advantageous for controlled DNA delivery.

3.2. Micro- and nanosphere DNA encapsulation

Micro- and nanosphere DNA encapsulation results are plotted in Figs. 3–6 as a function of polyanhydride content. As seen in these plots, successful DNA encapsulation (commonly reported as ≥20% (Perez et al., 2001; Little et al., 2004)), using this double emulsion/solvent evaporation procedure, occurs only at low polyanhydride copolymer ratios. Figs. 3 and 4 represent poly(L-lactic acid-*co*-sebacic anhydride):poly(β-amino ester) micro- and nanospheres, respectively. For poly(L-lactic acid-*co*-sebacic anhydride):poly(β-amino ester) microspheres, DNA encapsulation drops to nearly 0% at 10% polyanhydride content; poly(L-lactic acid-*co*-sebacic anhydride):poly(β-amino ester) nanospheres show a similar pattern with low levels (0–10%) of DNA encapsulation observed at 5 and 10% polyanhydride content. Figs. 5 and 6 rep-

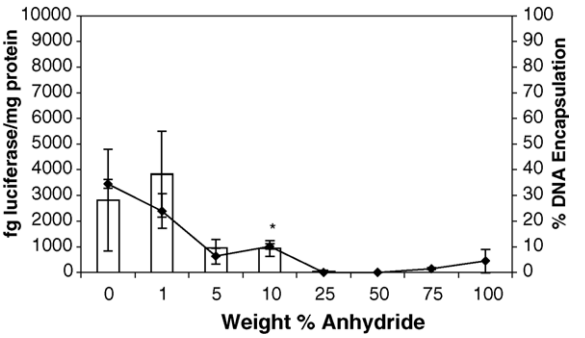

Fig. 4. Transfection (bars; left axis; 24 h time point) and gene encapsulation (data points; right axis) data for poly(L-lactic acid-*co*-sebacic anhydride):poly(β-amino ester) nanospheres. The asterisk (*) denotes statistical significance ($p < 0.05$) between the measured transfection levels at the same weight% anhydride content compared to Fig. 6.

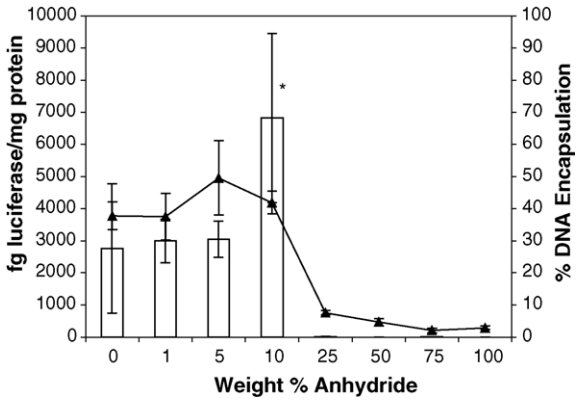

Fig. 5. Transfection (bars; left axis; 24 h time point) and gene encapsulation (data points; right axis) data for poly(L-lactic acid-*co*-adipic anhydride):poly(β-amino ester) microspheres. The asterisk (*) denotes statistical significance ($p < 0.05$) between the measured transfection levels at the same weight% anhydride content compared to Fig. 3.

resent poly(L-lactic acid-*co*-adipic anhydride):poly(β-amino ester) micro- and nanosphere DNA encapsulation. This copolymer showed DNA encapsulation extended to 10% polyanhydride content for microspheres and between 10 and 25% polyanhydride content for nanospheres.

These results illustrate an interesting relationship between copolymer synthesis and formulation and pCMV-Luc plasmid DNA encapsulation. Previous efforts at encapsulating pCMV-Luc using only poly(sebacic anhydride):poly(β-amino ester) double emulsion/solvent evaporation formulations consistently showed 0–5% DNA encapsulation; whereas, PLGA:poly(β-amino ester) formulations demonstrated between 20 and 70% encapsulation efficiency (unpublished observations). We hypothesized that the hydrophobic nature of poly(sebacic-anhydride) was leading to poor DNA encapsulation using this formulation technique. To overcome this and still provide anhydride groups for potential surface modification, poly(ester-anhydride) copolymers (based upon sebacic acid monomeric units) were synthesized. Observable DNA encapsulation (>5%) occurred at low polyanhydride copolymer ratios (0–5% polyanhydride content for microspheres and 0–10% for nanospheres). To further extend encapsulation over a wider copolymer range, copolymers containing the less hydrophobic poly(adipic anhydride) were synthesized. DNA encapsulation was now possible at higher polyanhydride contents (0–10% for microspheres and 0–25% for nanospheres) supporting the notion that polyanhydride hydrophobicity negatively affects hydrophilic DNA encapsulation using the double emulsion/solvent evaporation formulation technique.

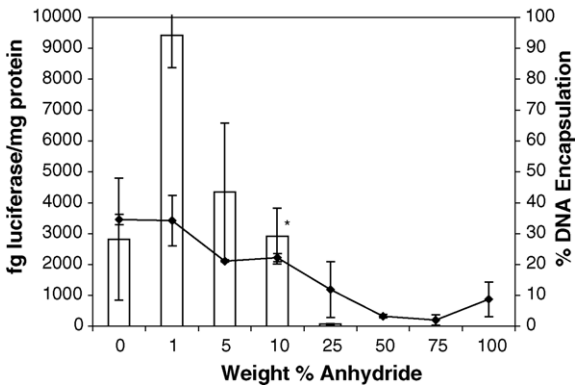

Fig. 6. Transfection (bars; left axis; 24 h time point) and gene encapsulation (data points; right axis) data for poly(L-lactic acid-*co*-adipic anhydride):poly(β-amino ester) nanospheres. The asterisk (*) denotes statistical significance ($p < 0.05$) between the measured transfection levels at the same weight% anhydride content compared to Fig. 4.

3.3. *In vitro transfection assay*

A macrophage cell line (P388D1) was tested for micro- and nanosphere mediated gene delivery. Throughout this report, transfection success is considered relative to prior efforts using polymeric microspheres for gene delivery to the P388D1 cell line (Hedley et al., 1998; Little et al., 2004). To facilitate comparisons to previous work (Little et al., 2004), 25% (w/w) poly(β-amino ester) was incorporated into the spheres, and though other amounts were tested (unpublished results), 25% poly(β-amino ester) showed maximum transfection results and was subsequently used in this study. For poly(L-lactic acid-*co*-sebacic anhydride):poly(β-amino ester) microspheres (Fig. 3), transfection closely followed DNA encapsulation. Transfection peaked at ∼5000 fg luciferase/mg protein with a copolymer ratio of 1% poly(sebacic anhydride). For nanospheres (Fig. 4), similar transfection units were obtained with ∼4000 fg luciferase/mg protein again observed at 1% poly(sebacic anhydride) content.

By using adipic acid as a polyanhydride precursor, transfection was provided over a wider copolymer range. This is first observed in Fig. 5 where microspheres containing 10% poly(adipic anhydride) copolymer extended luciferase transfection (∼7000 fg luciferase/mg protein) compared to sebacic anhydride copolymer microspheres (Fig. 3). Fig. 6 shows maximum transfection data (∼9000 fg luciferase/mg protein) with 1% poly(adipic anhydride) nanospheres, and when compared to Fig. 4, transfection is extended to 10% poly(adipic anhydride) copolymer levels. Statistical analysis (Student's *t*-test) was performed between pairs of data points for Figs. 3–6. Each microsphere data point (0, 1, 5, 10, 25, 50, 75, 100% anhydride level) was directly compared between Figs. 3 and 5. Similarly, Figs. 4 and 6 were compared for nanosphere transfection. Data points statistically different (the 10% anhydride level) were denoted and the results help illustrate extended transfection at higher overall polyanhydride content using adipic acid-derived copolymers.

Though data were collected for 24, 48, and 72 h, transfection trends mirrored those of the 24-h time point, and no obvious transfection increase was observed over this time. Therefore, only the 24-h time point results are presented. Furthermore, results presented are for 10 μg/ml micro- and nanosphere dosages due to increased levels of toxicity (measured through BCA-protein quantification) observed for the 50 μg/ml dosage (84% cellular protein [averaged for all 10 μg/ml dosages] versus 55% [50 μg/ml dosages, with the higher dosage likely leading to an increased acidic environment as the spheres degraded] when compared to untreated controls). To ensure that gene delivery was facilitated by micro- and nanospheres, transfection was tested using unencapsulated DNA with no measurable luciferase production observed.

Micro- and nanosphere transfection and DNA encapsulation correlate. Both were observed at low polyanhydride content, and transfection levels are comparable to previous reports either with or without the inclusion of poly(β-amino ester) (Hedley et al., 1998; Little et al., 2004). For example, PLGA microspheres show a 10–100 fg/mg transfection level; whereas, PLGA(75%):poly(β-amino ester) (25%) microspheres show transfection between 10,000 and 50,000 fg luciferase/mg protein (Little et al., 2004). The current data suggest that the less hydrophobic poly(adipic anhydride) copolymers facilitate better gene encapsulation and improved transfection at higher overall anhydride levels.

4. Conclusion

These results illustrate the plasticity of this class of poly(ester-anhydride) copolymers (regarding both synthesis and formulation) and how this flexibility can be used to influence gene encapsulation and transfection. Specifically, copolymers containing low polyanhydride ratios led to micro- and nanospheres with gene encapsulation and transfection properties comparable to previous literature reports. The results were systematically improved by using different polyanhydrides (adipic for sebacic anhydride). This success shows promise for future gene delivery applications using these copolymers.

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