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Degradable Cationic Shell Crosslinked Knedel-like Nanoparticles: Synthesis, degradation, nucleic acid binding and *in vitro* evaluation

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Abstract

In this work, degradable cationic shell crosslinked knedel-like (deg-cSCK) nanoparticles were developed as an alternative platform to replace similar non-degradable cSCK nanoparticles that have been utilized for nucleic acids delivery. An amphiphilic diblock copolymer poly(acrylamidoethylamine)₉₀-*block*-poly(DL-lactide)₄₀ (PAEA₉₀-*b*-PDLLA₄₀) was synthesized, self-assembled in aqueous solution and shell crosslinked using a hydrolyzable crosslinker to afford deg-cSCKs with an average core diameter of 45 ± 7 nm. These nanoparticles were fluorescently labeled for *in vitro* tracking. The enzymatic- and hydrolytic-degradability, siRNA binding affinity, cell uptake and cytotoxicity of the deg-cSCKs were evaluated. Esterase-catalyzed hydrolysis of the nanoparticles resulted in the degradation of *ca.* 24% of the PDLLA core into lactic acid within 5 d, as opposed to only *ca.* 9% degradation from aqueous solutions of the deg-cSCK nanoparticles in the absence of enzyme. Cellular uptake of deg-cSCKs was efficient, while exhibiting low cytotoxicity with LD₅₀ values of *ca.* 90 μ g/mL and 30 μ g/mL in RAW 264.7 mouse macrophages and MLE 12 cell lines, respectively, *ca.* 5–6-fold lower than the cytotoxicity observed for non-degradable cSCK analogs. Additionally, deg-cSCKs were able to complex siRNA at an N/P ratio as low as 2, and were efficiently able to facilitate cellular uptake of the complexed nucleic acids.

Keywords

Poly(lactide); degradable; nanoparticle; nucleic acid delivery; cellular uptake; cytotoxicity

1. Introduction

Recent studies have shown significant progress in the development of cationic polymeric nanoparticles that can facilitate cellular entry and delivery of nucleic acids, with promise toward gene therapy and improving therapeutic outcomes of currently available medications. Nucleic acid delivery is a multi-step process that involves a number of extra- and intracellular barriers in which inefficiencies at any stage may result in dramatic changes in their overall effectiveness.^{1–3} While nucleic acids are poorly taken up by cells and rapidly cleared from the circulatory system, due to their size, negative charge and instability in the

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presence of serum nucleases,¹ they can be optimized to remain longer in the circulation, shielded against undesirable biological interactions and protected from enzymatic degradation *via* electrostatic complexation to cationic nanoparticles.^{2,4}

Although cationic nanoparticles are a promising class of nucleic acid delivery agents, they vary significantly in their effectiveness and cytotoxicity, depending on the characteristics of the nanocarrier and cell type being administered. We have previously developed cationic shell crosslinked knedel-like (cSCK) nanoparticles composed of poly(acrylamidoethylamine)_n-*b*-polystyrene_m copolymers, that have been shown to efficiently transfect mammalian cells with both antisense oligonucleotides, siRNA and plasmid DNA.⁵⁻⁹ When a portion of the primary amines on the shell of these cSCKs was replaced with tertiary amines, the transfection efficiency was increased and cytotoxicity decreased in HeLa cells.⁹ Recently, the block copolymer precursor of these cSCKs was modified to bear histamines to facilitate endosomal escape of the nanoparticles.⁷ Furthermore, these cSCKs were templated onto anionic cylinders to form hierarchical assemblies as a multifunctional platform for theranostic applications.⁸ While these robust cSCKs with tunable properties hold great potential as nucleic acid delivery agents, the inherent cytotoxicity, immunogenicity and other adverse biological responses associated with long term accumulation of these non-degradable polymers in the body after repeated administration presents a major complication to their clinical applications.

Synthetic delivery vectors that are composed of biodegradable material are advantageous over the non-degradable counterparts, mainly due to their potential to degrade over time and release the therapeutic payload triggered by disassembly of the particles, which in-turn facilitates clearance of the nanoparticles from the body and improves the safety profile for patients. In the design of nucleic acid delivery vectors, a multitude of efforts has been made to overcome many of the extra- and intracellular obstacles by incorporation of hydrophilic poly(ethylene glycol) moieties to suppress the adsorption of serum proteins,¹⁰⁻¹² membrane destabilizing and pH-sensitive polymers for endosomal escape,^{7,13-15} targeting ligands¹⁶⁻¹⁸ or covalent crosslinking for stabilization against counter polyion exchange.^{8,19-21} However, only a few studies have been focused on minimizing the overall toxicity of cationic carriers by integration of degradability and evaluating their long term fate. Among the recently developed cationic degradable synthetic platforms, acid-sensitive acetalated-dextran microparticles have shown tunable degradation rates with rapid release of plasmid under endosomal conditions,²² poly(β -amino ester)-containing polyelectrolyte multilayers have been utilized for controlled release and surface-mediated delivery of nucleic acids,^{23,24} and both polyphosphoester- and polycarbonate-based cationic nanocarriers have exhibited minimal cytotoxicities at relatively high polymer concentrations.²⁵⁻²⁷

In this study, our primary focus was to incorporate the multifunctionality and high stability of the previously developed cSCKs^{5,7,9} into a degradable analog, and to confirm that these deg-cSCKs maintain the ability to bind nucleic acids and enhance their intracellular bioavailability. Therefore, deg-cSCKs have been designed by the replacement of polystyrene with hydrolyzable poly(DL-lactide) within the core domain, and the use of cleavable ester-based crosslinkers throughout the shell region, while maintaining a similar poly(acrylamidoethylamine) shell composition. Based on our findings from the enzymatic-hydrolysis of poly(L-lactide)-containing anionic SCKs,²⁸ this study also extends the synthetic versatility of the poly(lactide)-containing SCK system, by converting the anionic shell to possess cationic characteristics for nucleic acid complexation. The enzymatic-and hydrolytic-degradability of deg-cSCKs were analyzed and their cellular uptake and cytotoxicity were evaluated *in vitro*. Additionally, the abilities of the deg-cSCKs to complex and deliver negatively-charged nucleic acids (siRNA as a model drug) intracellularly were evaluated.

2. Materials and methods

2.1. Materials

Azobisisobutyronitrile (AIBN, 98%, Aldrich) was recrystallized from methanol before use. DL-lactide (98%, Alfa Aesar) was purified by recrystallization from ethyl acetate. Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dichloromethane (DCM), tetrahydrofuran (THF), toluene, trifluoroacetic acid (TFA), porcine liver esterase (PLE) (activity = 154 U/mg, concentration = 35.6 mg of protein/mL) 1,8-diazabicycloundec-7-ene (DBU) and *N*-ethylpiperidine hypophosphite (EHP) were used as received from Sigma-Aldrich Company, St. Louis, MO. The amine reactive cleavable crosslinker ethylene glycol bis(succinimidylsuccinate) was purchased from Thermo Fisher Scientific and the amine-reactive fluorescent label Alexa Fluor® 647 carboxylic acid, succinimidyl ester was purchased from Life Technologies. Chain transfer agent (CTA) 5-hydroxypentyl 2-(((dodecylthio)carbonothioyl)thio)-2-methylpropanoate and tert-butyl (2-methacrylamidoethyl)carbamate monomer were synthesized as reported.^{29,30} Spectro/Por® membranes (MWCO 12–14 kDa, Spectrum Medical Industries, Inc., Laguna Hills, CA) were used for dialysis. The lactate colorimetric assay kit (ab65331) was purchased from Abcam®.

2.2. Instruments

¹H NMR and ¹³C NMR spectra were recorded on Varian 500 MHz and Varian 300 MHz spectrometers. Chemical shifts were referenced to solvent resonance signals.

The DMF gel permeation chromatography (GPC) was conducted on a Waters Chromatography, Inc. (Milford, MA) system equipped with an isocratic pump model 1515, a differential refractometer model 2414, and a four-column set of 5 µm guard (50 × 7.5 mm), Styragel HR 45 µm DMF (300 × 7.5 mm), Styragel HR 4E 5 µm DMF (300 × 7.5 mm), and Styragel HR 2 5 µm DMF (300 × 7.5 mm). The system was equilibrated at 70 °C in prefiltered DMF containing 0.05 M LiBr, which served as polymer solvent and eluent (flow rate set to 1.00 mL/min). Polymer solutions were prepared at a concentration of *ca.* 3 mg/mL, and an injection volume of 200 µL was used. Data collection and analysis were performed with Empower 2 v. 6.10.01.00 software (Waters, Inc.). The system was calibrated with poly(ethylene glycol) standards (Polymer Laboratories, Amherst, MA).

Thermogravimetric analysis (TGA) was performed under N₂ atmosphere using a Mettler-Toledo model TGA/SDTA851e, with a heating rate of 10 °C/min and cooling rate of 5 °C/min. Measurements were analyzed using Mettler-Toledo Star® v. 7.01 software.

Glass transition temperatures (*T*_g) were measured by differential scanning calorimetry (DSC) on a Mettler-Toledo DSC822® (Mettler-Toledo, Inc., Columbus, OH), with a heating rate of 10 °C/min. Measurements were analyzed using Mettler-Toledo Star® v. 7.01 software. The *T*_g was taken as the midpoint of the inflection tangent, upon the third heating scan.

Dynamic light scattering (DLS) measurements were conducted using Delsa Nano C (Beckman Coulter, Inc., Fullerton, CA) equipped with a laser diode operating at 658 nm. Size measurements were made in nanopure water (*n* = 1.3329, *η* = 0.890 cP at 25 ± 1 °C). Scattered light was detected at 165° angle and analyzed using a log correlator over 70 accumulations for a 3.0 mL sample in a glass sizing cell (4.0 mL capacity). The samples in the glass sizing cell were equilibrated for 30 minutes before measurements were made. The photomultiplier aperture and the attenuator were automatically adjusted to obtain a photon counting rate of *ca.* 10 kcps. Calculation of the particle size distribution and distribution averages was performed using CONTIN particle size distribution analysis routines. The

peak averages of histograms from number distributions out of 70 accumulations were reported as the average diameters of the particles.

Transmission electron microscopy (TEM) images were collected on a JEOL 1200EX operating at 100 kV and micrographs were recorded at calibrated magnifications using a SIA-15C CCD camera. The samples as aqueous solutions (4 μ L) were deposited onto carbon-coated copper grids. Excess sample was wicked off using filter paper and the grids were allowed to dry in air for 2 min. Following that, the grids were stained with 4 μ L of a 2% uranyl acetate aqueous solution. Excess stain was wicked off using filter paper after 15 sec. The sample grids were dried under vacuum overnight before analyses.

A SpectraMax M5 microplate reader was used for the analysis of lactic acid by the colorimetric assay.

2.3. Synthesis of poly(DL-lactide)₄₀-macro chain transfer agent (PDLLA₄₀-macroCTA)

A 250 mL Schlenk flask equipped with a stir bar was flame dried under vacuum and cooled under nitrogen. The flask was then charged with hydroxyl-functionalized chain transfer agent (390 mg, 0.868 mmol), DL-lactide (5.01 g, 34.7 mmol) and DCM (100 mL), and stirred under nitrogen until complete dissolution of monomer. A stock solution of DBU in DCM (158 mg in 100 μ L, 1.04 mmol) was added under nitrogen flow and the yellow color solution was allowed to stir at room temperature. After 45 min >99% monomer to polymer conversion was achieved as measured by ¹H NMR, and the reaction mixture was quenched by the addition of acetic acid (1.04 g, 17.4 mmol) and poured into 150 mL of methanol to afford a yellow color sticky precipitation. The reaction mixture was concentrated by rotary evaporation of dichloromethane. The precipitate was isolated by filtration and dried under vacuum to yield 3.8 g (70% yield based on conversion) of yellow color powder. ¹H NMR (CDCl₃, ppm): δ 5.25-5.08 (m, CH of PDLLA), 4.17-4.01 (m, CH₂CH₂O and COOCH₂ of CTA), 3.28-3.20 (t, CH₂CH₂S of CTA), 1.75-1.61 (m, CH₃CCH₃ and OCH₂(CH₂)₃CH₂O of CTA), 1.61-1.42 (m CH₃ of PDLLA), 1.42-1.16 (m, CH₃(CH₂)₁₀CH₂S of CTA), 0.88 (t, terminal CH₃ of CTA). ¹³C NMR (CDCl₃, ppm): δ 169.6, 69.2, 16.8. IR (cm⁻¹): 2994-2947, 1746, 1452, 1381, 1265, 1182, 1082, 866. M_n^{calc} = 6200 Da, $M_n^{DMF-GPC}$ = 16100 Da, PDI = 1.04. DSC: T_g = 41 °C. TGA in N₂: 260–385 °C, >92% mass loss.

2.4. Synthesis of poly(acrylamidoethylamine-boc)₉₀-block-poly(DL-lactide)₄₀ (P(AEA-boc)₉₀-b-PDLLA₄₀ diblock copolymer

A 50 mL Schlenk flask equipped with a stir bar was flame dried under vacuum and charged with PDLLA₄₀-macroCTA (1.50 g, 0.242 mmol), tert-butyl (2-methacrylamidoethyl)carbamate (5.17 g, 24.2 mmol), DMF (33 mL), AIBN (7.94 mg, 48.4 μ mol) and, stirred under nitrogen. Upon complete dissolution of the starting material, the reaction mixture was subjected to three freeze-pump-thaw cycles. The reaction was allowed to stir for 6.5 h at 70 °C to afford 90% conversion. The product was precipitated into a 1:1 methanol: water mixture, isolated by filtration and dried under vacuum overnight to yield 4.4 g (73% yield) of pale yellow color solid. ¹H NMR (DMF-d₇, ppm): δ 8.02-7.78 (br, boc-NH), 6.99-6.71 (br, CH₂CH₂NHCO), 5.32-5.24 (m, CH of PDLLA), 4.37-3.96 (br, CH₂CH₂O, COOCH₂ and CH₂CH₂S of CTA), 3.47-3.05 (br, NHCH₂CH₂NH), 2.40-2.00 (br, CH of PAEA-boc backbone), 1.86-1.63 (br, CH₂ of PAEA-boc backbone and, CH₃CCH₃ and OCH₂(CH₂)₃CH₂O of CTA), 1.61-1.51 (m CH₃ of PDLLA), 1.45-1.35 (br, CH₃ of boc groups), 1.35-1.20 (br, CH₃(CH₂)₁₀CH₂S of CTA), 0.88 (t, terminal CH₃ of CTA). ¹³C NMR (DMF-d₇, ppm): δ 175.4 (br), 170.1 (br), 156.4, 78.4, 69.6-69.1 (br), 45.6-39.0 (br), 28.8, 16.9. IR (cm⁻¹): 3312, 3078-2976, 1755, 1690-1651, 1520, 1452, 1366, 1250, 1167, 1090, 1001. M_n^{calc} = 25500 Da, $M_n^{DMF-GPC}$ = 27000 Da, PDI = 1.26. DSC: T_g

= 48 °C. TGA in N₂: 200 – 250 °C, 39% mass loss; 250 – 440 °C, 33% mass loss; 28% mass remaining above 440 °C.

2.5. Synthesis of poly(acrylamidoethylamine)₉₀-block-poly(DL-lactide)₄₀ (PAEA₉₀-b-PDLLA₄₀) diblock copolymer and micelles

The diblock copolymer P(AEA-boc)₉₀-b-PDLLA₄₀ (100 mg, 3.92 μmol) was dissolved in excess TFA (6.03 g, 52.9 mmol) in a 20 mL scintillation vial and allowed to stir for 2 h at room temperature. After this reaction period, TFA was removed under vacuum, to yield a light yellow solid. The product was dissolved in DMF (30 mL), transferred into a presoaked dialysis tube (12–14 kDa MWCO) and dialyzed against nanopure water for 2 d with frequent replacement of the dialysis medium with fresh nanopure water, to yield 84.6 mL of clear micelles in water (0.80 mg/mL). DLS: $D_{h(n)} = 103 \pm 24$ nm, $D_{h(v)} = 125 \pm 48$ nm, $D_{h(i)} = 193 \pm 72$ nm. TEM: $D_{av} = 43 \pm 8$ nm. Zeta potential = +59 mV (in nanopure water at pH 5–6). From the solution of micelles, ca. 20 mL was lyophilized for characterization purposes. ¹H NMR (DMF-d₇, ppm): δ 8.86–8.34 (br, NH), 6.61–4.48 (br, NH₂), 5.32–5.24 (m, CH of PDLLA, overlapped with br NH₂ peaks), 4.37–3.96 (br, CH₂CH₂O, COOCH₂ and CH₂CH₂S of CTA, overlapped with br NH₂ peaks), 3.74–3.34 (br, NH₂CH₂), 3.31–3.07 (br, CH₂NHCO), 2.44–2.09 (br, CH of PAEA backbone), 1.91–1.60 (br, CH₂ of PAEA backbone and, CH₃CCH₃ and OCH₂(CH₂)₃CH₂O of CTA), 1.60–1.47 (m, CH₃ of PDLLA), 1.35–1.20 (br, CH₃(CH₂)₁₀CH₂S of CTA), 0.88 (t, terminal CH₃ of CTA). ¹³C NMR (DMSO-d₆, ppm): δ 176.0–174.0 (br), 169.6, 80.2–78.6 (multiple overlapping peaks), 69.1, 43.8–41.2 (br), 17.1. IR (cm⁻¹): 3590–2502 (br), 1749, 1643, 1537, 1454, 1389, 1179, 1126, 835, 799. $M_n^{calc} = 16500$ Da, DSC: $T_g = 48$ °C. TGA in N₂: 200 – 245 °C, 25% mass loss; 245 – 450 °C, 52% mass loss; 24% mass remaining above 450 °C.

2.6. Preparation of deg-cSCK and labeling with Alexa Fluor 647

For the preparation of deg-cSCKs, the pH of a solution of micelles (63.5 mL, 50.8 mg, 3.08 μmol) was adjusted to 8–9 by the addition of an aqueous solution of 1 M Na₂CO₃ under stir, and a stock solution of ethylene glycol bis[succinimidylsuccinate] (6.93 mg, 15.2 μmol) in 100 μL of DMF was added to the solution of micelles at room temperature, and the reaction mixture was allowed to stir. After 30 min, 11 mL (8.80 mg, 0.533 μmol) of the reaction mixture was transferred into a separate vial for the dye conjugation. A stock solution of Alexa Fluor® 647 carboxylic acid, succinimidyl ester (0.334 mg, 0.267 μmol) in DMF was added to the 11 mL reaction mixture, and protected from light. Both the reaction mixtures were allowed to stir for an additional 2.5 h. For the removal of unreacted crosslinker and free dye, both reaction mixtures were transferred into presoaked dialysis tubing (12–14 kDa MWCO) and dialyzed against nanopure water for 2 d in two separate beakers, with frequent replacement of the dialysis medium with fresh nanopure water to yield deg-cSCKs and Alexa Fluor 647-labeled deg-cSCKs. For unlabeled deg-cSCKs, DLS: $D_{h(n)} = 107 \pm 27$ nm, $D_{h(v)} = 134 \pm 46$ nm, $D_{h(i)} = 193 \pm 72$ nm. TEM: $D_{av} = 45 \pm 7$ nm. Zeta potential = +55 mV (in nanopure water at pH 5–6). For labeled deg-cSCKs, dye/polymer = 0.25, as quantified by UV-vis spectroscopy. Based on the measured final concentration of the deg-cSCKs (0.85 mg/mL for unlabeled deg-cSCK and 0.92 mg/mL for labeled deg-cSCKs), the aqueous solutions were divided into aliquots and lyophilized in 1.5 mL centrifugation tubes (0.5 mg polymer/tube), and stored at –4 °C. All measurements were repeated by resuspension of lyophilized deg-cSCKs in 0.1 M Tris-HCl buffer at pH 7.4 (1.0 mL, 0.80 mg/mL). Both D_h and D_{av} were similar to the measurements obtained in nanopure water, however, zeta potential was less positive, +25 mV in 0.1 M Tris-HCl buffer at pH 7.4.

2.7. Degradation of cSCK nanoparticles

For the degradation experiments, fresh solutions of nanoparticles were prepared by dissolution of lyophilized deg-cSCKs in 0.1 M Tris-HCl buffer containing 0.05% w/v NaN₃

at pH 7.4 (1.0 mL, 0.80 mg/mL) in 1.5 mL centrifugation tubes. For the enzyme-catalyzed hydrolysis of deg-cSCKs, esterase (4 μ L) was added to each tube, stirred and incubated at 37 °C. To determine the hydrolytic degradation rates of the samples in the absence of enzyme catalysis, 4 μ L of Tris-HCl buffer was added to each solution (to maintain identical polymer concentrations as the enzyme catalyzed experiment), and incubated at 37 °C. The lactate assays were performed following the standard protocol of ab65331 as described below. At time points 0, 1, 3 and 5 d, 50 μ L of sample was withdrawn from each tube, mixed with 50 μ L of lactate enzyme assay mix in a 100 μ L Falcon clear well, protected from light and incubated at room temperature for 30 min to produce color, and analyzed using a plate reader for absorption at 450 nm. Each analysis was performed in triplicates and average absorbance values with standard deviations were reported. A calibration curve for DL-lactic acid was constructed by the use of serial dilutions produced from a standard solution of 0.1 M DL-lactic acid in 0.1 M Tris-HCl buffer (calibration range: 0–10 nmol of lactate), and the production of DL-lactic acid at each time point was quantified and reported as a percentage of the total theoretical DL-lactic acid present in each solution at 0.80 mg/mL of nanoparticles.

2.8. Cell culture

RAW 264.7 and MLE 12 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7, a mouse macrophage cell line, was cultured in RPMI medium 1640 (Cellgro, Mediatech, Manassas, VA) supplemented with 10% fetal calf serum (Sigma-Aldrich) and 2 mM L-glutamine. MLE 12, a cell line with features of alveolar type II cells,³¹ was cultured in Ham's F12 (Cellgro) supplemented with 1% of Insulin-Transferrin-Sodium Selenite (100X, Sigma-Aldrich, St. Louis, MO), 10 nM hydrocortisone (BD Bioscience, San Jose, CA), 10nM β -estradiol (Sigma-Aldrich), 10 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Cellgro), 2 mM L-glutamine (Cellgro), and 2% fetal bovine serum (Sigma-Aldrich).

2.9. Immunostaining and microscopy

Cells were seeded (3×10^4 cells/dish) on MatTek dishes (MatTek Corporation, Ashland, MA) that were previously coated with filter-sterilized type I rat tail collagen 50 μ g/mL (BD Bioscience) in 0.02 N acetic acid. Cells incubated with labeled deg-cSCKs were washed with PBS, fixed with 4% PFA, and then stained for actin using phalloidin labeled with Alexa Fluor 488 (dilution, 1:40, Life Technologies, Grand Island, NY). Epifluorescent images were captured using a Leica DM5000 microscope (Wetzlar, Germany) with a Retiga 200R camera interfaced with QCapture Pro software (Q Imaging, Surrey, BC, Canada). All photomicrographs were globally adjusted for contrast and brightness using Photoshop (Adobe Systems, San Jose, CA). Cells were scanned along the z-axis to collect 0.5 μ m thick sections. Fluorescence and differential interference contrast (DIC) images were overlaid. All photomicrographs were globally adjusted for contrast and brightness using Photoshop (Adobe Systems, San Jose, CA).

RAW 264.7 mouse macrophages (1×10^5 cells/well) were plated in glass-bottom six-well plate (MatTek Co., Ashland, MA) in Dulbecco's Modified Eagle Medium (DMEM) (10% fetal bovine serum and 1% penicillin/streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h to adhere. Then, the medium was replaced with a fresh media 1-h prior to the addition of siRNA-loaded cSCKs (200 nM final concentration of the 5'-Cy3-siRNA). The cells were incubated with the formulation for 3 h and washed extensively with PBS. Then, DRAQ-5 (Biostatus Ltd., Shephed, Leicestershire, UK) was utilized to stain the nucleus (30 min incubation, followed by extensive washing with PBS). Cells were then fixed with 1% formaldehyde for 20 min, washed once with PBS. The cells were then stored in 1 mL PBS in the refrigerator and analyzed by laser scanning

confocal microscopy (LSCM) (LSM 510, Zeiss, Jena, Germany). The images were collected under the same conditions (laser power, detector gain, *etc.*) for consistency, and $\lambda_{\text{excitation}}$ of 543 and 633 nm were utilized for the Cy3 and DRAQ-5, respectively.

2.10. Flow cytometry

Flow cytometry was used to quantify cell entry of labeled deg-cSCKs. Cells were seeded (5×10^4 cells/well) on 48-well plates, and incubated with the labeled nanoparticles. Cells were washed three times with flow cytometry buffer composed of phosphate buffered saline (PBS pH 7.4), and 2% fetal bovine serum. Replicate samples were analyzed (10,000 events per sample) using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). A sample of cells that was not incubated with nanoparticles was used as a negative control. The percent cells expressing Alexa Fluor 647 and median fluorescent intensity (MFI) was determined using CELLquest software (BD Biosciences).

2.11. Cell viability

Cells were seeded (2.5×10^4 cells/well) on black frame, clear bottom 96-well plates (BD Falcon, Franklin Lakes, NJ). Labeled deg-cSCKs were added to cells then incubated at 37°C, 5% CO₂, for 24 h. Cells were then equilibrated to room temperature for 30 min and washed three times with PBS. Cell-Titer-Glo Reagent (Promega, Madison, WI) was added to cells and mixed on an orbital shaker for 2 min at room temperature. Cells were then incubated for an additional 10 min at room temperature and analyzed for luminescence using a Molecular Devices SPECTRAmax Gemini Microplate Spectrofluorometer (Sunnyvale, CA).

2.12. Gel shift assay

Agarose gels (1%) were prepared in Tris-acetate-EDTA buffer (Bio-Rad Laboratories, Inc., Hercules, CA). The siRNA (5'-Cy3-(sense strand)-GGCCACAUCGGAUUUCACU, $M_w = 13814$ g/mol, Dharmacon, Chicago, IL), either free or complexed to the degradable cSCKs at nitrogen-to-phosphate (N/P) ratios ranging from 1–10 (1.3 μ g siRNA/25 μ L/well, Tris buffer, 10 mM, pH 7.4), were mixed with glycerol (20% v/v) prior to the electrophoresis. Gel electrophoresis was carried out using a horizontal apparatus at 100 V for 30 min and fluorescence imaging of the separated siRNA bands was performed using a ChemiDoc XRS (Bio-Rad Laboratories, Inc.).

3. Results and discussion

In the design of deg-cSCKs, our goal was to replace core and crosslinker portions of the previously reported non-degradable cSCK constituents with hydrolyzable ester moieties (Figure 1), and evaluate their physicochemical properties and biological performance, with comparison to the non-degradable analog. Deg-cSCK nanoparticles were constructed by the self-assembly of a novel amphiphilic diblock copolymer PAEA₉₀-*b*-PDLLA₄₀, followed by crosslinking between the amine functionalities presented within the shell by the addition of an amine-reactive ethylene glycol bis(succinimidylsuccinate) ester crosslinker containing a cleavable ester linkage in the middle. Our design of the amphiphilic diblock copolymer PAEA₉₀-*b*-PDLLA₄₀ precursor to the cSCKs incorporates a hydrophobic, degradable PDLLA segment and a hydrophilic PAEA segment that provides cationic character in its protonated form, and built-in functionality. The amine functionalities presented in the hydrophilic segment were utilized for crosslinking as well as fluorophore labeling for *in vitro* tracking.

The compositions and structures of the new deg-cSCK and the previously reported non-degradable analog are compared in Figure 1. Each has an amphiphilic core-shell

morphology, provided by the supramolecular assembly of diblock copolymers into micelles, followed by stabilization *via* crosslinking of polymer block segments within the shell. The non-degradable cSCK was composed of a hydrophobic polystyrene core, and a hydrophilic cationic poly(acrylamidoethylamine) shell that was 5% crosslinked with amide-containing crosslinkers (Figure 1A). As the precursor diblock copolymer of the non-degradable cSCK was synthesized by atom transfer radical polymerization (ATRP), the chain end of the hydrophilic segment consisted of an ethyl propanoate group. In the deg-cSCK nanoparticle design, while the hydrophobic polystyrene core segment was substituted with enzymatically- and hydrolytically-degradable poly(DL-lactide), the hydrophilic poly(acrylamidoethylamine) shell composition was maintained, and 5% crosslinked with hydrolyzable ester-containing linkers, as shown in Figure 1B. Additionally, since different polymerization mechanisms were required, the PDLLA-containing amphiphilic diblock copolymer consisted of a dodecyl trithiocarbonate as the hydrophilic chain end group, significantly different from its non-degradable counterpart.

Sequential ring opening polymerization (ROP) and reversible addition-fragmentation chain transfer (RAFT) polymerization were employed to obtain the initial PDLLA₄₀ homopolymer and subsequent P(AEA-boc)₉₀-*b*-PDLLA₄₀ diblock copolymer (Scheme 1A). The degrees of polymerization and well-defined structures for the polymers were confirmed by a combination of ¹H NMR spectroscopy and GPC. Assuming full retention of the trithiocarbonate chain end, ¹H NMR spectra allowed for the determination of the degree of polymerization of PDLLA by comparing the unique terminal methyl protons of the CTA resonating at 0.88 ppm with the broad methyl and methine proton signals of PDLLA from 1.42–1.61 ppm and 5.08–5.25 ppm, respectively. Additionally, the degree of polymerization of the P(AEA-boc) segment was calculated based on both conversion and end group analysis from ¹H NMR spectroscopy, by comparing the PDLLA methine proton signal to that of the methine protons of AEA-boc from 3.47–3.05 ppm. GPC analyses of the isolated polymers showed mono-modal molecular weight distributions with polydispersity indices (PDI) less than 1.3 (Scheme 1B), indicating the controlled nature of the polymerization process. Deprotection of P(AEA-boc)₉₀-*b*-PDLLA₄₀ was accomplished by stirring with excess TFA for 2 h at room temperature with no additional solvent being required. Quantitative removal of the boc protecting groups was evidenced by loss of signals from boc-related protons (¹H NMR, DMF-d₇, 1.45–1.35 ppm).

The deg-cSCKs were prepared by the aqueous supramolecular assembly of the PAEA₉₀-*b*-PDLLA₄₀ block copolymers into micelles, followed by stabilization *via* crosslinking of AEA units along the PAEA blocks within the shell (Scheme 2). Cationic micelles were assembled by evaporation of TFA from the amphiphilic diblock copolymer PAEA₉₀-*b*-PDLLA₄₀ and dilution in DMF, followed by dialysis against nanopure water for 2 d to remove excess TFA and DMF. The micelles were then subjected to a nominal crosslinking density of 5% throughout the shell region by the addition of a di-*N*-hydroxysuccinimide (NHS)-activated acrylic acid crosslinker to a stirring solution of micelles at pH 8–9 to yield deg-cSCKs. After 3 h, the solution of deg-cSCKs was dialyzed against nanopure water for 2 d to remove the NHS byproducts, and non-conjugated crosslinkers. The crosslinking density (xlinking) refers to the percentage of amine residues that were theoretically consumed during the crosslinking reaction.

When the average core diameters (D_{av}) and hydrodynamic (D_h) diameters of the deg-cSCKs were analyzed by TEM and DLS, respectively, the overall dimensions of the deg-cSCKs were significantly larger than those of their non-degradable analogs. As shown in Figure 2A, core diameters of the deg-cSCKs in the dry state were 45 ± 7 nm, and the monomodal size distributions observed by DLS were consistent with the TEM measurements with hydrodynamic diameters of $D_{h(n)} = 107 \pm 27$ nm, $D_{h(v)} = 134 \pm 46$ nm and $D_{h(i)} = 193 \pm 72$

nm (Figure 2B). As summarized in Table 1, the average core diameters and number-average hydrodynamic diameters of the deg-cSCKs were *ca.* 5 times greater than the non-degradable cSCKs. Furthermore, the zeta potentials of the deg-cSCKs were highly positive, 55 mV as opposed to only 21–26 mV of the non-degradable cSCKs in nanopure water at pH 5–6.

As the overall dimensions of the deg-cSCKs were considerably larger than the non-degradable analog, several synthetic parameters were explored to determine whether tailoring the composition of the precursor diblock copolymer PAEA₉₀-*b*-PDLLA₄₀, would in-turn affect its aqueous assembly behavior, to afford assemblies in the ~15 nm range. Our first approach was to increase the hydrophilic to hydrophobic ratio of the amphiphilic diblock copolymer. Therefore, a series of polymers with varying block copolymer lengths was synthesized, self-assembled in nanopure water, and their hydrodynamic diameters were analyzed by DLS. As summarized in Table 2, two polymers were synthesized with increasing hydrophilic PAEA lengths of 180 and 270 repeat units (polymers 2 and 3, respectively), while maintaining the same PDLLA length of 40 repeat units as the original diblock copolymer (polymer 1). Another diblock copolymer was synthesized with approximately half the hydrophobic PDLLA length with 20 repeat units, while maintaining a similar hydrophilic PAEA length of 90 repeat units (polymer 4). Interestingly, when these diblock copolymers were assembled into micelles by direct dissolution in nanopure water, the micelles that originated from polymer 2 were uniform and similar in hydrodynamic diameters ($D_{h(n)} = 105 \pm 28$) to the original micelles prepared from polymer 1, and in contrast to our expectations, the assemblies prepared from polymer 3 were larger in their hydrodynamic diameters ($D_{h(n)} = 237 \pm 32$). Additionally, assembly attempt of polymer 4 (PAEA₉₀-*b*-PDLLA₂₀) resulted in a cloudy suspension. Therefore, although the replacement of non-degradable polystyrene for degradable PDLLA was initially considered to be a simple approach, the nanoparticle production from different polymer materials provided unexpected outcomes and challenges.

Our next approach was to evaluate the role of the hydrophobic dodecyl trithiocarbonate end group on the self-assembly behavior of the amphiphilic diblock copolymer. Considering the hydrophobic dodecyl trithiocarbonate group at the hydrophilic terminus might provide another hydrophobic unit, so that the AB diblock copolymer could act essentially as an ABC triblock, the dodecyl chain of the protected diblock copolymer P(AEA-boc)₉₀-*b*-PDLLA₄₀ was removed by radical-induced reduction of the trithiocarbonate group by reacting with AIBN and EPHP at 100 °C in toluene for 2 h.³² Reduction of the dodecyl trithiocarbonate group was evident by the color change from yellow to colorless within 1 h of reaction. After 2 h, the polymer was isolated by precipitation into hexane and quantitative removal of the dodecyl chain end was evidenced by loss of signals from the terminal methyl protons (¹H NMR, DMSO-*d*₆, 0.88 ppm). The diblock copolymer was then deprotected, and assembled into micelles. From both TEM and DLS analyses, the core and hydrodynamic diameters of the cationic micelles formed in the absence of the dodecyl chain remained unchanged at *ca.* 45 nm and *ca.* 100 nm, respectively, confirming our earlier work that showed the diameters of the SCKs prepared from poly(methylacrylate)₈₂-*b*-poly(*N*-(acryloyloxy)succinimide)_{0.29-co-(N-acryloylmorpholine)}_{0.71})₁₅₅ being relatively unaffected in the presence and absence of the dodecyl group at the hydrophilic end.³³ Furthermore, to determine if the relatively larger sizes of the degradable cationic assemblies were an outcome of the self-assembly technique rather than a property of the precursor polymer, the diblock copolymer PAEA₉₀-*b*-PDLLA₄₀ was assembled into micelles by several selective-solvent displacement techniques from DMF to water, DMSO to water and THF to water, and to eliminate possible counterion effects caused by the presence of excess TFA, the micelles were dialyzed against an acidic aqueous solution of 0.1 M HCl and then 0.1 M Tris-HCl buffer at pH 7.4 for 2 d, and analyzed for sizes. Despite the several efforts focused on reducing the sizes of the cationic nanostructures, by optimizing the length, composition and assembly methodologies

of the diblock copolymers, the dimensions of the cationic assemblies either remained unchanged or were larger than the sizes obtained from the original unmodified diblock copolymer PAEA₉₀-*b*-PDLLA₄₀.

Although incorporation of degradability to the cSCK nanoparticle has been one of our primary design features, rapid disassembly of the nanoparticles was not desired, because *in vitro* and *in vivo* studies are time consuming and rapid physicochemical degradation would pose challenges where prior disintegration of the defined morphologies may occur. Based on our finding of proteinase K-catalyzed core hydrolysis of PLA-containing anionic SCK nanoparticles,²⁸ the newly developed cationic SCKs were incubated in the presence of a more biologically-relevant enzyme, porcine liver esterase, and the generation of the degradation product DL-lactic acid was analyzed using a lactate colorimetric assay. As expected, the enzyme-catalyzed cSCK nanoparticles showed accelerated release of DL-lactic acid (*ca.* 24% of its original PDLLA content), as opposed to only *ca.* 9% core hydrolysis for the uncatalyzed system, within 5 d of core degradation (Figure 3). Although hydrolytic degradation of the deg-cSCKs was fairly slow, minor changes in physicochemical properties due to core and crosslinker degradation of the nanoparticles may cause considerable variations in their biological behavior. Therefore, to increase the shelf-life of the deg-cSCKs storage methodologies were developed. Immediately following the synthesis of deg-cSCKs by crosslinking of micelles and dialysis against nanopure water, the aqueous solutions were aliquoted into centrifugation tubes, lyophilized (0.5 mg of polymer/tube, based on final concentration of the deg-cSCKs) and stored at -4 °C. These lyophilized deg-cSCKs were resuspended in 0.1 M Tris-HCl buffer at pH 7.4 and their physicochemical characteristics were extensively reexamined by DLS, TEM and zeta potential measurements. We found that all measurements were similar to the original characterization data. Therefore, the deg-cSCKs were routinely stored in the lyophilized form, and suspended into aqueous solution immediately before *in vitro* experiments.

The capability of deg-cSCKs to enter murine cell lines was determined by confocal microscopy, for Alexa Fluor 647-labeled deg-cSCKs. After 1 h of cell incubation, deg-cSCKs were observed within the cytoplasm of both the macrophage-like RAW 264.7 and the epithelial MLE 12 cell lines. Nanoparticles were most abundant as clusters within the cytoplasm shown by images obtained in the horizontal (x,y) in RAW 264.7 cells (Figure 4, A1 and A2) and MLE 12 cells (Figure 4, B1 and B2). In both cell lines, internalization of deg-cSCK was further confirmed in reconstructed vertical axis images (x,z; Figure 4, A3 and B3). Nanoparticles were also observed within the region of the cell membrane, most notable within the MLE 12 cells, identified by actin cytoskeletal staining with phalloidin (Figure 4B1).

To determine the distribution and efficiency of deg-cSCK uptake in both RAW 264.7 and MLE 12 cells, we also obtained images using low power epifluorescent microscopy. One hour after delivery, nearly all of the cells contained deg-cSCKs (Figure 5 A and B). Additionally, there was a difference in the distribution of deg-cSCK uptake among cells, such that some cells contained large amounts of particles (Figure 5, panels A1 and B1). These observations suggest that the uptake of deg-cSCK in both cell types was rapid and efficient.

To quantify the efficiency and variability of uptake over time, fluorescently-labeled deg-cSCK nanoparticles of different concentrations were incubated with cell lines and analyzed using flow cytometry (Figure 6). Analysis of the median fluorescence intensity in each sample suggested that there was a greater uptake of the particles at higher concentrations and at 24 h compared to 1 h (Figure 6A). Cell uptake at 1 and 24 h of deg-cSCK was both concentration- and time-dependent in both cell lines (Figure 6B). In both cells, efficient cell

uptake of deg-cSCK was achieved with low nanoparticle concentration (at 1 h, 0.3 $\mu\text{g/mL}$ and at 24 h, 0.03 $\mu\text{g/mL}$).

Degradable cSCKs cellular entry was associated with low cell cytotoxicity. To determine the viability of cells, each cell line was incubated with various concentrations of deg-cSCKs and analyzed by measuring cell ATP activity after 24 h (Figure 7). In each cell line, viability remained high until a LD50 concentration of 30 $\mu\text{g/mL}$ in MLE 12 and 90 $\mu\text{g/mL}$ in RAW 264.7, indicating higher resistance of RAW 264.7 cells to toxicity. Additionally, in RAW 264.7 cells, the deg-cSCKs were 5–6 times less toxic with LD50 values *ca.* 90 $\mu\text{g/mL}$ compared to their non-degradable counterparts with LD50 values *ca.* 16 $\mu\text{g/mL}$.⁷

The ability of the deg-cSCKs to complex negatively-charged nucleic acids (siRNA as a model drug) and to deliver them intracellularly were tested by a gel shift assay and a confocal fluorescence microscopy assay with RAW 264.7 mouse macrophages. The nanoparticles were able to complex the siRNA at a ratio of deg-cSCK ammonium groups to siRNA phosphodiester groups (N/P ratio) of 2 (Figure 8A). In agreement with the behavior of the non-degradable cSCK analogs,⁷ deg-cSCK were able to efficiently facilitate the cellular uptake of the complexed siRNA as seen in the confocal microscopy images of the cells treated with the siRNA/deg-cSCKs (siRNA and nucleus appear in red and blue, respectively) (Figure 8B, B3 and B4), as compared to the control-untreated cells (Figure 8B, B1 and B2). The uptake of the siRNA complexed to the nanoparticles is consistent with the cellular uptake of the nanoparticles themselves, as tested by fluorescence microscopy and flow cytometry (Figures 4, 5 and 6).

4. Conclusions

In summary, we have reported fundamental advances in the synthetic methodologies for the preparation of degradable, functionalizable cationic nanoparticles, together with their siRNA binding affinity and biological evaluation *in vitro*. Specifically, we have synthesized a degradable analog of the previously established cSCKs by incorporation of hydrolyzable ester linkages throughout the hydrophobic core region and shell crosslinkers. Although the diameters of the deg-cSCKs were considerably greater than their non-degradable analogs, the deg-cSCKs were able to efficiently bind siRNA at a low N/P ratio of 2, and facilitate their cellular entry. The rates of esterase-catalyzed hydrolysis of the PDLLA core of the deg-cSCKs were greater than for uncatalyzed hydrolysis conditions. With the cell internalization efficiencies, low cytotoxicities, and ability to carry siRNA, the future work will investigate the intracellular trafficking of the degradable cSCKs, and compare their characteristics and transfection efficiencies to the standard non-degradable cSCKs that have been utilized extensively *in vitro* and *in vivo*.

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Figure 1.

Chemical structures superimposed on the nanostructures of (A) previously reported non-degradable cSCK composed of poly(acrylamidoethylamine)_n-*b*-polystyrene_m ($n = 130$, $m = 40$ or $n = 160$, $m = 30$) with an ethyl propanoate chain end, 5% shell crosslinked ($p = 0.05$) using an amide-containing crosslinker,^{5,7} and (B) newly developed degradable cSCK composed of poly(acrylamidoethylamine)₉₀-*b*-poly(DL-lactide)₄₀ with a dodecyl trithiocarbonate chain end, 5% shell crosslinked ($q = 0.05$) with an ester-containing crosslinker.

Figure 2. Characterization of deg-cSCKs by (A) TEM; $D_{av} = 45 \pm 7$ nm, and (B) DLS histograms of number-, volume- and intensity-averaged hydrodynamic diameters; $D_{h(n)} = 107 \pm 27$ nm, $D_{h(v)} = 134 \pm 46$ nm, $D_{h(i)} = 193 \pm 72$ nm.

Figure 3.
Degradation profiles of deg-cSCKs in the presence and absence of porcine liver esterase in pH 0.1 M Tris-HCl buffer (pH 7.4) at 37 °C within 5 d.

Figure 4.

Confocal images of cells stained with actin marker phalloidin (green), after incubation with Alexa Fluor 647-labeled deg-cSCK (red) for 1 h, showing intracellular localization of deg-cSCK nanoparticles in (A) mouse macrophage RAW 264.7 and (B) alveolar epithelial MLE 12 cell lines. The image axis is indicated (x,y and x,z). In panels A1-2 and B1-2 (x,y), fluorescent and differential interference contrast (DIC) images are overlaid on the right. Panels A3 and B3 (x,z), show particles within a single cell. Scale bars = 10 μm .

Figure 5.

Epifluorescence microscopy images of deg-cSCKs (red) incubated in (A) RAW 264.7 mouse macrophages and (B) MLE 12 cells. Cells were stained with actin marker phalloidin (green) and the nuclei were stained with DAPI (blue). Scale bar = 10 μm .

Figure 6.

Concentration- and time-dependent deg-cSCK cell uptake in RAW 264.7 and MLE 12 cells incubated with Alexa Fluor 647-labeled deg-cSCKs at the indicated concentrations for 1 h and 24 h, analyzed by flow cytometry. (A) Median fluorescence intensity (MFI). (B) Cell uptake. Shown are the mean $\pm$ SD of triplicate samples representative of two independent experiments.

Figure 7.

The effect of degradable cSCK on cell viability. RAW 264.7 (red) and MLE 12 (blue) cells were incubated with Alexa Fluor 647-labeled deg-cSCK at the indicated concentrations for 24 h, then analyzed for cell viability using an assay based on the luminescent detection of ATP. Cell viability is expressed relative to the ATP activity in untreated control cells (100%). Shown are the average cell viability values with standard deviations of triplicate samples, representative of two independent experiments.

Figure 8.

(A) Gel-shift assay of Cy3-labeled siRNA, either naked ($N/P = 0$) or complexed to the degradable cSCKs at increasing N/P ratios. (B) Laser scanning confocal microscopy images of RAW 264.7 mouse macrophages that were either untreated (B1 and B2), or treated with Cy3-labeled siRNA (200 nM) complexed with the degradable cSCKs at N/P ratio of 5 (B3 and B4) for 3 h. The Cy3-siRNA and the nucleus stained with DRAQ-5 appear in the red (upper left) and blue (lower left) panels, respectively. The light transmitted images (upper right) and merged images (lower right) are also presented. B2 and B4 images correspond to the three-dimensional images of the untreated cells and cells treated with the Cy3-siRNA/cSCKs complexes, respectively.

Scheme 1.

(A) Synthesis of PDLLA_{40} homopolymer and $\text{P(AEA-boc)}_{90}\text{-}b\text{-PDLLA}_{40}$ diblock copolymer by sequential ROP and RAFT polymerization, followed by acidolysis to afford the amphiphilic diblock copolymer $\text{PAEA}_{90}\text{-}b\text{-PDLLA}_{40}$. (B) Size exclusion chromatography traces of the homopolymer and protected diblock copolymer samples, showing narrow molecular weight distributions.

Scheme 2.

Preparation of deg-cSCK nanoparticles by self assembly of amphiphilic diblock copolymer PAEA₉₀-*b*-PDLLA₄₀ followed by crosslinking (top), and enzymatic hydrolysis of the deg-cSCKs by degradation of the PDLLA core and cleavable crosslinker. TC = dodecyl trithiocarbonate group.

Table 1

Comparison of physicochemical characteristics (size and surface charge) of non-degradable and degradable cSCKs.

Parameter	Non-degradable	Degradable
D _{av} by TEM (nm)	~ 9 ^a ~ 10 ^b	~ 45
D _h by DLS (nm)	D _{h(v)} ~ 14 ^a D _{h(n)} ~ 21 ^b D _{h(v)} ~ 30 ^b D _{h(i)} ~ 106 ^b	D _{h(n)} ~ 107 D _{h(v)} ~ 134 D _{h(i)} ~ 193
Zeta potential pH 5–6 (mV)	+21 ^a +26 ^b	+55

Non-degradable cSCKs prepared from poly(acrylamidoethylamine)_n-*b*-polystyrene_m with DPs

^a n = 130, m = 40 and,

^b n = 160, m = 30.

Table 2

Summary of PAEA_y-*b*-PDLLA_x diblock copolymer compositions synthesized with varying degrees of polymerization (DPs), determined by ¹H NMR spectroscopy, molecular weights (*M_n*) and PDIs, determined from GPC, and hydrodynamic diameters (*D_h*) of resulting aqueous micellar assemblies, determined by DLS.

Polymer	DP (¹ H NMR)	<i>M_n</i> (Da) (GPC)	PDI (GPC)	<i>D_h</i> (nm) (DLS)
1	y = 90 x = 40	27000	1.26	<i>D_{h(n)}</i> = 101 ± 26 <i>D_{h(v)}</i> = 128 ± 46 <i>D_{h(i)}</i> = 191 ± 74
2	y = 180 x = 40	45900	1.33	<i>D_{h(n)}</i> = 105 ± 28 <i>D_{h(v)}</i> = 139 ± 56 <i>D_{h(i)}</i> = 231 ± 102
3	y = 270 x = 40	47300	1.35	<i>D_{h(n)}</i> = 237 ± 32 <i>D_{h(v)}</i> = 250 ± 36 <i>D_{h(i)}</i> = 266 ± 38
4	y = 90 x = 20	23100	1.29	Cloudy suspension