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Clusterin antisense complexed with chitosan for controlled intratumoral delivery

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Abstract

The purpose of this work was to characterize an injectable, intratumoral, controlled release delivery system for clusterin antisense oligonucleotide (clusterin ASO) based on clusterin ASO complexed with chitosan microparticles (CC complexes) and blended with a biodegradable polymeric paste (CC in paste). The effect of clusterin ASO/chitosan ratio on the physicochemical properties of CC complexes and the influence of chitosan and polymeric paste on the *in vitro* release and stability of clusterin ASO were investigated. Chitosan had an intrinsic pK_a of 6.2. Chitosan particles incubated at different pHs swelled to approximately 600% of their dry weight and had a mean diameter of approximately 200 μm. As the amount of chitosan in CC complexes was increased or as the pH was decreased, zeta potentials became increasingly less negatively charged and the amount of clusterin ASO complexed with chitosan increased. Clusterin ASO released into PBS or plasma *in vitro* from polymeric paste and CC in paste in a similar manner with a burst phase of release followed by a slow sustained release. The ratio of clusterin ASO to chitosan and incorporation into polymeric paste influenced the rate and extent of clusterin ASO release. Inclusion of clusterin ASO with or without chitosan in polymeric paste inhibited the *in vitro* degradation of clusterin ASO in plasma. Treatment of PC-3 cells *in vitro* with clusterin ASO alone or clusterin ASO released from the various formulations resulted in 52–62% inhibition of the expression of clusterin protein. Degradation studies showed that approximately 40% of the full-length clusterin ASO remained from both clusterin ASO alone and CC complex samples when incubated in 50% plasma *in vitro* for 4 days. In conclusion, the amount of clusterin ASO loaded into microparticulate chitosan was dependent on the amount of chitosan present and the pH of the environment and clusterin ASO released from the various formulations in a controlled manner and in a bioactive form.

Keywords: Oligonucleotide delivery; Controlled drug delivery; Chitosan; Polymeric paste; Clusterin antisense; Prostate cancer

1. Introduction

Therapeutic oligonucleotides, including small interfering ribonucleic acids, ribozymes and antisense (ASOs), are expected to provide treatments against a number of different diseases (Tomari and Zamore, 2005; Yu et al., 2005; So et al., 2005). Unfortunately the development of therapeutic oligonucleotides in the clinic has been slowed due to a number of drug delivery issues. These include poor targeting of oligonucleotides to the site of action, rapid elimination from the body, degradation by nucleases and inefficient cellular uptake (Brignole et al., 2003; Vinogradov et al., 2004; Wang et al., 2003). Methods used to improve cellular uptake of oligonucleotides and decrease their rate of degradation by nucleases include modifying the chemical backbone and side groups of oligonucleotides, preparing oligonucleotide conjugates, and complexing oligonucleotides with cationic or polycationic moieties including lipids, amino acids and polymers (Nakajima et al., 2000; Allerson et al., 2005; Juliano, 2005; Oupický et al., 2000).

Several mechanisms have been proposed to explain the decreased protein expression caused by ASO inhibition of

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messenger ribonucleic acid (mRNA) translation following hybridization. These include hybrid cleavage, translational arrest, inhibition of splicing, and inhibition of 5′-cap formation (Crooke, 1999; Stahel and Zangemeister-Wittke, 2003). Clusterin protein is involved in a number of different cancers and Gleave et al. have demonstrated that clusterin antisense oligonucleotide (clusterin ASO) targeted at clusterin mRNA has the ability to down-regulate the expression of clusterin protein in prostate cancer, bladder cancer and renal cell carcinoma (Jones and Jomary, 2002; Gleave and Miyake, 2005; Duggan et al., 2002; Miyake et al., 2002).

There are very few studies of the controlled release of oligonucleotides from polymer matrices. De Rosa et al. incorporated oligonucleotides into poly(lactide-*co*-glycolide) (PLGA) microspheres in the absence or presence of polyethyleneimine and demonstrated controlled release of the oligonucleotides *in vitro* over 30 days (De Rosa et al., 2002). Other groups showed that oligonucleotides incorporated into PLGA microspheres were released *in vitro* in a sustained manner for 35–56 days (Lewis et al., 1998; Hussain et al., 2002). In addition, the degradation of oligonucleotides *in vitro* in serum was inhibited when loaded into PLGA microspheres (Lewis et al., 1998). Our research group is studying the intratumoral, controlled delivery of drugs, including clusterin ASO, in solid prostate tumors (Springate et al., 2005). An injectable and biodegradable polymeric paste consisting of a triblock copolymer (triblock copolymer) blended with methoxy-poly(ethylene glycol) (MePEG) at a w/w ratio of 40/60, was developed for intratumoral administration and controlled release of paclitaxel. The addition of low molecular weight MePEG to the triblock copolymer resulted in a blended "polymeric paste" that could easily be injected by syringe through a 22 gauge needle. In aqueous media or a tissue environment, the paste set to a semi-solid implant at 37 °C (Jackson et al., 2004). This intratumoral formulation was effective at inhibiting the growth of human prostate tumors grown in mice when loaded with 10 mg of paclitaxel (Jackson et al., 2000). In a subsequent study, clusterin ASO was complexed within chitosan microparticles (CC complexes) and incorporated into the polymeric paste (CC in paste) with 1 mg of paclitaxel or docetaxel. Intratumoral injection of the formulations in PC-3 and LNCaP human prostate cancer xenograft murine models resulted in reduced mean tumor volume by greater than 50% and reduced mean serum prostate specific antigen (PSA) level by more than 70% (Springate et al., 2005).

Chitosan is a biocompatible, biodegradable polysaccharide composed of *N*-acetylglucosamine and *N*-D-glucosamine monomers. The amine on the *N*-D-glucosamine residues is protonated at a lower pH, producing a polycationic polymer that has been shown to effectively condense and deliver plasmids/genes (high molecular weight DNA) *in vitro* and *in vivo* (Renbutsu et al., 2005; Freier et al., 2005; Mi et al., 2002; Ishii et al., 2001; Ramos et al., 2005). Chitosan and its derivatives have been complexed with polyanionic genes and antisense oligonucleotides in acidic solutions to increase *in vitro* transfection efficiency and *in vivo* efficacy (Gao et al., 2005; MacLaughlin et al., 1998). The chitosan solution is then mixed with genes or oligonucleotides to form complexes. Due to cooperative electrostatic interactions between the polycationic chitosan and the polyanionic DNA these mixtures resulted in polyelectrolyte complexes or coacervated complexes with mean diameters generally between 100 and 1500 nm (Erbacher et al., 1998; Mao et al., 2001; Gao et al., 2005).

To our knowledge, although chitosan has been used to increase DNA and oligonucleotide transfection and efficacy (Gao et al., 2005; MacLaughlin et al., 1998), chitosan has not been used to control the release of oligonucleotides from polymer-based implants or depot formulations. The goal of our work was to prepare a delivery system that localizes clusterin ASO at the site of action, protects the ASO from degradation, and provides controlled release of the ASO. Polyanionic clusterin ASO was loaded into polycationic chitosan microparticles by a solvent loading process to form CC complexes. Unlike the colloidal or coacervate complexes formed from chitosan and oligonucleotide solutions, CC complexes were formed using microparticulate chitosan that swelled as the clusterin ASO solution was absorbed into the microparticles. CC complexes were dried and incorporated into the triblock copolymer/MePEG polymeric paste by blending to form CC in paste. The objectives of these studies were to investigate the influence of clusterin ASO to chitosan ratio on the physicochemical properties of CC complexes, the release characteristics of clusterin ASO from CC complexes and paste formulations, and the stability of clusterin ASO in CC complexes and paste formulations in human plasma *in vitro*.

2. Materials and methods

2.1. General chemicals, polymers and oligonucleotides

Hydrochloric acid, sodium hydroxide, acetonitrile and 70% isopropyl alcohol were obtained from Fisher Scientific (USA). Sodium heparin human plasma was obtained from Bioreclamation Inc. (USA). Cell culture materials were obtained from Invitrogen Incorporation (USA). Western blotting materials were obtained from Bio-Rad (USA). All other chemicals were obtained from Sigma Chemical Co. (USA). Chitosan (degree of deacetylation of 82–87%) was obtained from Carbomer, Inc. (USA). MePEG (molecular weight (MW) 350 g/mol) was obtained from Union Carbide (USA). Triblock copolymer of poly(D,L-lactide-random-caprolactone)-block-poly(ethylene glycol)-block-poly(D,L-lactide-random-caprolactone) was synthesized as previously described (Jackson et al., 2004). Pullulan MW standards were obtained from Polymer Labs Inc. (USA). All oligonucleotides used in this work were fully phosphorothioated. Clusterin ASO, 21-mer, sequence 5′-CAG CAG CAG AGT CTT CAT CAT-3′ was obtained from La Jolla (USA), TriLink BioTechnologies (USA) and the Nucleic Acid-Protein Service at The University of British Columbia (Canada) (NAPS). Clusterin ASO degradation products (20-, 19- and 18-mer sequences omitted units from the -CAT- 3′ end) and thymidine oligonucleotide internal standard, 25-mer, T25-3′ (T_{25}), were all from NAPS.

2.2. Preparation of CC complex and CC in paste formulations

Complexation data have been reported using a chitosan amine/DNA phosphate ratio and is based on the maximum theoretical charge of the chitosan amine groups (termed N/P or P/N ratio) (Ishii et al., 2001). However, few chitosan amines are protonated at physiological pH. Using a format similar to that employed by Lee et al., we report clusterin ASO/chitosan ratios on a w/w basis (Lee et al., 2001). Formulations were prepared as follows. Suspensions of chitosan microparticles were prepared in PBS at pH 6.6, 7.0 and 7.4, by vortexing chitosan (1 mg/mL) in 1 mL PBS for 10 s in a 2 mL Eppendorf tube. Clusterin ASO/chitosan microparticulate complexes, termed "CC complexes", were prepared by mixing clusterin ASO solution (1 mg/mL) to 1 mL of the appropriate concentration of chitosan suspension and vortexing for 10 s and equilibrating for 12 h. CC complexes with clusterin ASO/chitosan w/w ratios between 1/0.5 and 1/16 were prepared by pipetting the 2 mg/mL clusterin ASO solution onto the appropriate amount of chitosan powder in the corner of a 20 mL glass scintillation vial. The chitosan swelled in the aqueous clusterin ASO solution and the CC complexes were dried overnight. Clusterin ASO alone (no chitosan) was dried by pipetting the 2 mg/mL clusterin ASO solution into the corner of an empty glass scintillation vial and drying overnight. Polymeric paste was prepared in 1 g batches by blending 400 mg triblock copolymer and 600 mg MePEG in a 20 mL glass scintillation vial at 40 °C in a water bath. An appropriate amount of polymeric paste was added to dried CC complexes or clusterin ASO alone and blended with a spatula at 40 °C for 10 min to achieve homogeneous formulations. The paste containing the CC complexes was termed "CC in paste". For example a "CC in paste 1/6" contained 1, 6 and 43 mg of ASO, chitosan and paste, respectively. All formulations were stored at 4 °C.

2.3. Determination of chitosan intrinsic viscosity and molecular weight

The intrinsic viscosity $[\eta]$ of chitosan was determined using an Ubbelohde viscometer at 23 °C by constructing Huggins, Kraemer and Solomon–Ciuta plots after three measurements. Four chitosan solutions in a range of concentrations from 0.2 to 0.8% (w/v) in a solution of 0.25 mol/L acetic acid and 0.25 mol/L sodium acetate were prepared. The peak molecular weight (M_{GPC}) of chitosan was determined by gel permeation chromatography (GPC). Pullulan standards were prepared as a 0.1% (w/v) solution and assayed by GPC with a mobile phase of 0.25 mol/L acetic acid and 0.25 mol/L sodium acetate. The standards were eluted through Ultrahydrogel 2000 + 1000 columns (Waters, USA) joined in series. A GPC universal calibration curve was constructed by plotting the log of the product of the MW and the $[\eta]$ of the standards against the retention time of the peak of the standards. The MWs and the $[\eta]$ reference values for the pullulan standards were obtained from the supplier and Yomota et al. (1993), respectively. The M_{GPC} of chitosan was calculated from the GPC universal calibration curve.

2.4. Swelling and particle size analysis

Each sample consisted of approximately 10 mg chitosan powder accurately weighed into a 2 mL Eppendorf tube. Triplicate samples were suspended in 1 mL of PBS at pH 6.6, 7.0 or 7.4 and vortexed for 10 s. After 12 or 24 h the samples were centrifuged at 18,000× *g* for 5 min and the supernatant carefully withdrawn. The swollen chitosan microparticles were weighed and swelling reported as a percentage of dry weight. Chitosan alone and CC complexes containing 1 mg clusterin ASO and between 0.5 and 16 mg chitosan were suspended in 1 mL of PBS at pH 6.6, 7.0 or 7.4 and vortexed for 10 s immediately before analysis. Particle size distributions were determined in triplicate using a Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments, USA).

2.5. Intrinsic pK_a of chitosan

Chitosan suspensions were prepared by adding 10 mL of dilute solutions of sodium perchlorate with ionic strengths of 0, 0.01, 0.05, 0.075 or 0.1, to 10 mg of chitosan. To protonate the amine groups on chitosan microparticles, 20 μL of 10 mol/L hydrochloric acid was added to each suspension (allows for stoichiometric protonation of the chitosan amine groups). Potentiometric titration was performed by adding sodium hydroxide solution dropwise at a concentration of 0.1, 1 and/or 10 mol/L and converting to an equivalent amount of 0.1 mol/L sodium hydroxide solution. The intrinsic pK_a (pK_0) of chitosan was determined using a second degree polynomial best fit line of the pK_a versus ionic strength plot, and taking pK_0 to be the pK_a at an ionic strength of 0.

2.6. Zeta potential

The zeta potential of CC complexes was determined at different pH values. Chitosan alone and CC complexes containing 1 mg clusterin ASO and between 0.5 and 16 mg chitosan were suspended in a total of 10 mL of PBS at pH 6.6, 7.0 or 7.4. Samples were vortexed for 10 s immediately before analysis. Zeta potential measurements (in triplicate) were taken using a Zetasizer 3000 HS zeta potentiometer from Malvern Instruments (USA).

2.7. Complexation studies

Triplicate samples consisted of clusterin ASO alone and CC complexes containing 1 mg clusterin ASO and between 0.5 and 16 mg chitosan suspended for approximately 12 h in 2 mL of PBS at pH 6.6, 7.0 or 7.4. Samples were vortexed for 10 s and then centrifuged at 18,000× *g* for 5 min. Clusterin ASO concentrations in the supernatant (unbound fraction) were determined by absorbance at 260 nm.

2.8. In vitro release studies

The appropriate amount of each CC complex or CC in paste formulation ($n = 5$), containing 1 mg clusterin ASO, was incu-

bated with 1 mL of PBS at 37 °C with shaking. At various time points, the supernatant was removed for clusterin ASO analysis by high pressure liquid chromatography (HPLC) and replaced with fresh buffer at 37 °C. The remaining volumes of PBS release media were pooled and diluted to a concentration of 5 μmol/L clusterin ASO for treatment of PC-3 cells. After 28 days, the residual clusterin ASO remaining in the formulations in PBS was released by adding 3 mL of 50 mmol/L phosphate buffer at pH 11.0 to each replicate, followed by vortexing and sonicating. Studies were completed in a similar manner using human plasma ($n = 5$) in place of PBS with analysis of intact ASO and the 20-, 19- and 18-mer degradation products following extraction and desalting with analysis by capillary gel electrophoresis (CGE). After 28 days, the residual clusterin ASO remaining in the formulations in plasma was released by adding at least 5 mL strong anion exchange load/run buffer (10 mmol/L Tris–HCl, 0.5 mol/L potassium chloride, 20% (v/v) acetonitrile, pH 11.0) as per Section 2.10, followed by vortexing and sonicating.

2.9. HPLC

Anion exchange HPLC analysis of clusterin ASO was carried out similar to our previously reported study (Springate et al., 2005). The injection volume was 20 μL, detection was at 260 nm and the mobile phase was run at a constant 2 mL/min. Mobile phase A (0.01 mol/L Tris buffer pH 8, 0.005 mol/L sodium perchlorate and 40% (v/v) acetonitrile) was run for 2 min, followed by a linear gradient to mobile phase B (0.01 mol/L Tris buffer pH 8, 0.3 mol/L sodium perchlorate and 40% (v/v) acetonitrile) over 5 min, followed by 2 min of 100% mobile phase A.

2.10. ASO extraction and desalting from plasma

To prepare human plasma samples containing clusterin ASO and degradation products for analysis by CGE, an extraction and desalting method from plasma was developed from a method described by Leeds et al. (1996). AccuBond^{II} strong anion exchange (SAX) cartridges (Agilent Technologies, USA), were each equilibrated with acetonitrile, water and strong anion exchange load/run buffer. Each plasma sample was diluted with at least 5 mL strong anion exchange load/run buffer and spiked with T_{25} internal standard before vortexing and loading onto a SAX cartridge. The SAX cartridges were washed with 3 mL strong anion exchange load/run buffer. The oligonucleotides were eluted with 3 mL strong anion exchange elution buffer (10 mmol/L Tris–HCl, 0.5 mol/L potassium chloride, sodium bromide 1.0 mol/L, 30% (v/v) acetonitrile, pH 9.0) and then diluted with 6 mL dilution buffer (10 mmol/L Tris–HCl, 0.5 mol/L potassium chloride, sodium bromide 1.0 mol/L, pH 9.0). To desalt, Sep-Pak® Vac C_{18} cartridges (Waters, USA) were washed with acetonitrile, water and dilution buffer. The previously diluted eluent was loaded onto Vac C_{18} cartridges and washed with 5 mL water. The oligonucleotides were eluted with 3 mL of 20% (v/v) acetonitrile solution, dried down (nitrogen gas at 40 °C) and reconstituting with 100 μL water. The oligonucleotide solutions were applied to a nitrocellulose VS type filter (Millipore, USA) floating on 1 L of water and after 30 min, 75 μL was removed and diluted to 150 μL with water for assay by capillary gel electrophoresis (CGE).

2.11. CGE

Clusterin ASO and degradation products extracted and desalted from plasma were assayed by CGE using a P/ACE MDQ capillary electrophoresis system with an eCAP^{TM} DNA capillary, P/ACE MDQ UV–vis detector module and 32 Karat 5.0 software (Beckman Coulter, Inc., USA).

2.12. Tumor cell line and treatment of cells

A study was carried out to determine whether the clusterin ASO released into the PBS release media was bioactive *in vitro*. Human prostate cancer PC-3 cells were from the American Type Culture Collection (USA) and cultured in F-12 nutrient mixture. PC-3 cells (1×10^6) were plated into 10 cm diameter plates with 10 mL of PC-3 media per plate. One day later, release media containing clusterin ASO from the various formulations was incubated with 4 μg/mL Oligofectamine^{TM} for 20 min in serum-reduced Opti-Mem I before treating PC-3 cells in 10 cm plates at a final clusterin ASO concentration of 500 nmol/L. After 4 h, serum-containing media was added to the plates to increase the serum concentration to 10%. The cells were treated again 24 h later. Two days later the cells were harvested for clusterin protein analysis by Western blot ($n = 3$).

2.13. Western blots and densitometry

Samples containing 30 μg of protein from lysates of *in vitro* PC-3 cells were electrophoresed on a 1.5 mm SDS – 10% polyacrylamide gel and transferred to a BioTrace NT nitrocellulose membrane (Pall-Gelman Labs, USA) using a Mini-Protean III System with Mini Trans-blot module and a Power-Pac 300 power supply, all from Bio-Rad (USA). The filters were blocked overnight at 4 °C in TBST (2.42 g Tris base, 8 g sodium chloride, 3.8 mL of 1 mol/L hydrochloric acid, 1 g Tween®, made up to 1 L with distilled water) containing 5% non-fat milk powder, washed three times and then incubated for 1 h with a 1:500 diluted clusterin goat polyclonal antibody (Santa Cruz Biotechnology, Inc., USA) or 1:2000 diluted vinculin mouse monoclonal antibody (Sigma, USA). The membranes were then washed five times and incubated for 1 h with 1:5000 diluted horseradish peroxidase conjugated rabbit antigoat or goat antimouse antibody. Specific proteins were detected using an enhanced chemiluminescence ECL^{TM} Kit from GE Healthcare (USA). Protein expression was quantitated by densitometry using Fluor Chem^{TM} version 2.01 software (Alpha Innotech Corp., USA).

2.14. In vitro degradation of clusterin ASO

One milligram of clusterin ASO either alone or complexed with chitosan in a w/w ratio of clusterin ASO/chitosan of 1/6 was incubated in 1 mL of 50% plasma at 37 °C for 4 days. The use of 50% plasma in buffer allowed for the maintenance of a controlled pH. At various time points each sample was vortexed

and 100 μL of the suspension was withdrawn for oligonucleotide extraction, desalting and analysis by CGE.

2.15. Statistical analysis

Statistical analysis was by analysis of variance (ANOVA) using Microsoft Office Excel 2003 software.

3. Results

3.1. Chitosan intrinsic viscosity and molecular weight

The $[\eta]$ value for chitosan was determined from Huggins, Kraemer and Solomon–Ciuta plots and found to be $4.5 \pm 1.9\,\mathrm{dL\,g^{-1}}$ in 0.25 mol/L acetic acid and 0.25 mol/L sodium acetate solution at $23 \pm 1\,^{\circ}\mathrm{C}$. It was determined that chitosan had a broad MW distribution and an M_{GPC} of $360{,}000\,\mathrm{g\,mol^{-1}}$.

3.2. Swelling and particle size

Chitosan, incubated for 12 h in PBS at pH 6.6, 7.0 and 7.4, swelled to 630 ± 70, 660 ± 40 and $600 \pm 40\%$ of the dry chitosan weight, respectively. There was no difference in swelling between samples incubated at different pHs and no change in swelling was determined between 12 and 24 h incubation. Chitosan alone and CC complexes at the different pHs and different ASO/chitosan ratios had sizes ranging in diameter from approximately 10 to 850 μm with a mean diameter of approximately 200 μm.

3.3. Intrinsic pK_a (pK_0) of chitosan

The pK_a of chitosan ranged from 6.2 to 6.7 over ionic strengths ranging from 0 to 0.1 (sodium perchlorate concentrations of 0–0.1 mol/L). A best-fit line for the plot was determined using a second order polynomial equation of $y = 39.9x^2 - 8.92x + 6.21$. The pK_0 of chitosan was taken as the pK_a on this curve at an ionic strength of 0 and was determined to be 6.2.

3.4. Zeta potential

As shown in Fig. 1 the zeta potentials became increasingly less negatively charged as the amount of chitosan was increased in the CC complexes. At pHs 6.6 and 7.0, ASO/chitosan ratios of 1/4 or those containing greater amounts of chitosan had positive zeta potentials. At pH 7.4, only ASO/chitosan ratios of 1/7 or those containing greater amounts of chitosan had neutral or small positive zeta potentials.

3.5. Complexation studies

The amount of clusterin ASO complexed into the chitosan pellet was calculated as the difference between the total amount of clusterin ASO added and the amount of clusterin ASO determined to be in the supernatant. The difference in clusterin ASO uptake into chitosan microparticles between 12 and 24 h was equal to or less than 1.2%. As observed in Fig. 2 the amount of clusterin ASO in the pellet increased as the amount of chitosan was increased in the CC complexes. At pHs 6.6, 7.0 and

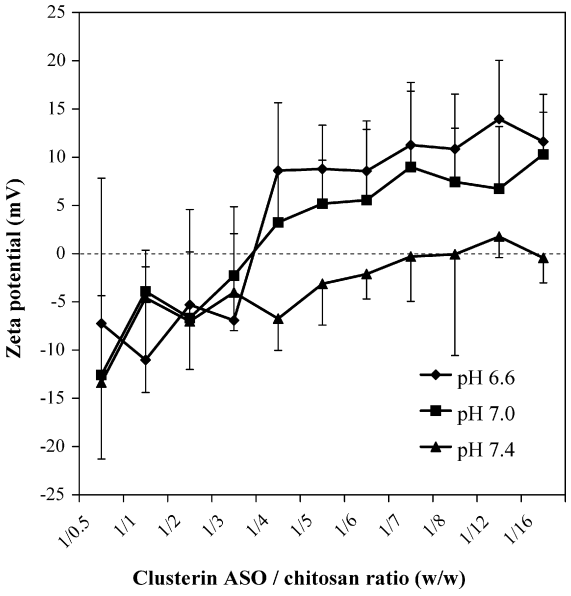

Fig. 1. Effect of clusterin antisense oligonucleotide (ASO)/chitosan ratio on the zeta potential values for clusterin ASO/chitosan microparticle complexes (CC complexes) buffered at different pHs. CC complexes containing 1 mg clusterin ASO and between 0.5 and 16 mg chitosan were suspended in 10 mL of PBS at pH 6.6, 7.0 or 7.4. Data points and error bars represent means ± standard deviation ($n = 3$).

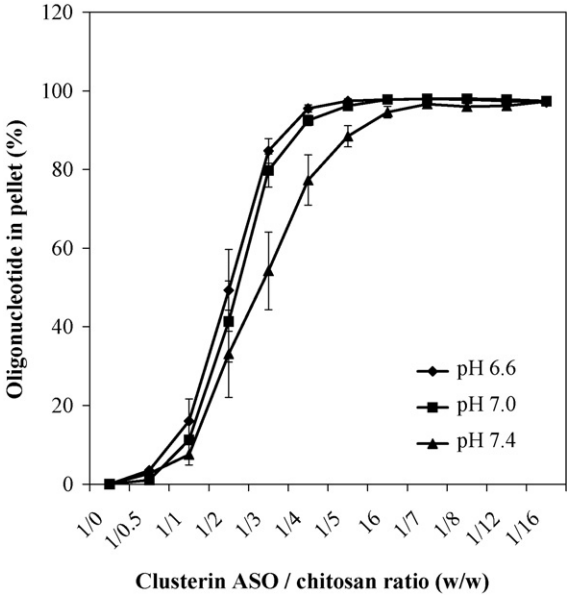

Fig. 2. Effect of clusterin antisense oligonucleotide (ASO)/chitosan ratio on the loading of clusterin ASO into chitosan microparticles (CC complexes) buffered at different pHs. CC complexes containing 1 mg clusterin ASO and between 0.5 and 16 mg chitosan were suspended in 2 mL of phosphate buffered saline at pH 6.6, 7.0 or 7.4, incubated overnight and centrifuged at $18{,}000\times g$ for 5 min to pellet the chitosan and associated clusterin ASO. The amount of clusterin ASO in the pellet was calculated by subtracting the amount of clusterin ASO in the supernatant from the total amount of clusterin ASO added. Data points and error bars represent means ± standard deviation ($n = 3$).

7.4, ASO/chitosan ratios of 1/4, 1/5 and 1/6, respectively, (or greater) were determined to have >95 ± 2% of the ASO in the chitosan pellet.

3.6. In vitro release profiles in PBS

Clusterin ASO released into PBS (pH 7.4) from polymeric paste and CC in paste in a biphasic manner, and from CC complexes in a triphasic manner (Fig. 3). The residual amounts of clusterin ASO in each formulation showed no ASO in the ASO in paste (no chitosan) sample and 30%, 56%, 57% and 59% remaining in the CC complex 1/2, 1/6 and CC complex/paste 1/2, 1/6 samples, respectively so that the total amount of clusterin ASO that could be accounted for (or recovered) on completion of the released studies ranged from 79 to 101% for the various formulations. When clusterin ASO was incubated alone in PBS a total of 91 ± 1% of the clusterin ASO was recovered on day 1. Clusterin ASO released from polymeric paste in a controlled manner over the first 7 days, followed by a slower release averaging 1 μg/day from day 7 to day 18, after which no clusterin ASO was detected in the release media for the remainder of the study (Fig. 3A). Clusterin ASO released from CC complexes in a rapid manner for the first 1–2 days, followed by a slower release averaging 1–4 μg per day for the next several days until days 5–6 (Fig. 3B). No clusterin ASO was detected from days 6 through 12, followed by clusterin ASO being released from days 12 to 28 at an average rate of 5 and 0.5 μg/day for clusterin ASO/chitosan ratios of 1/2 and 1/6, respectively. Clusterin ASO released from CC in pastes in a controlled manner over the first 4–5 days, followed by a slower release for the remainder of the 28

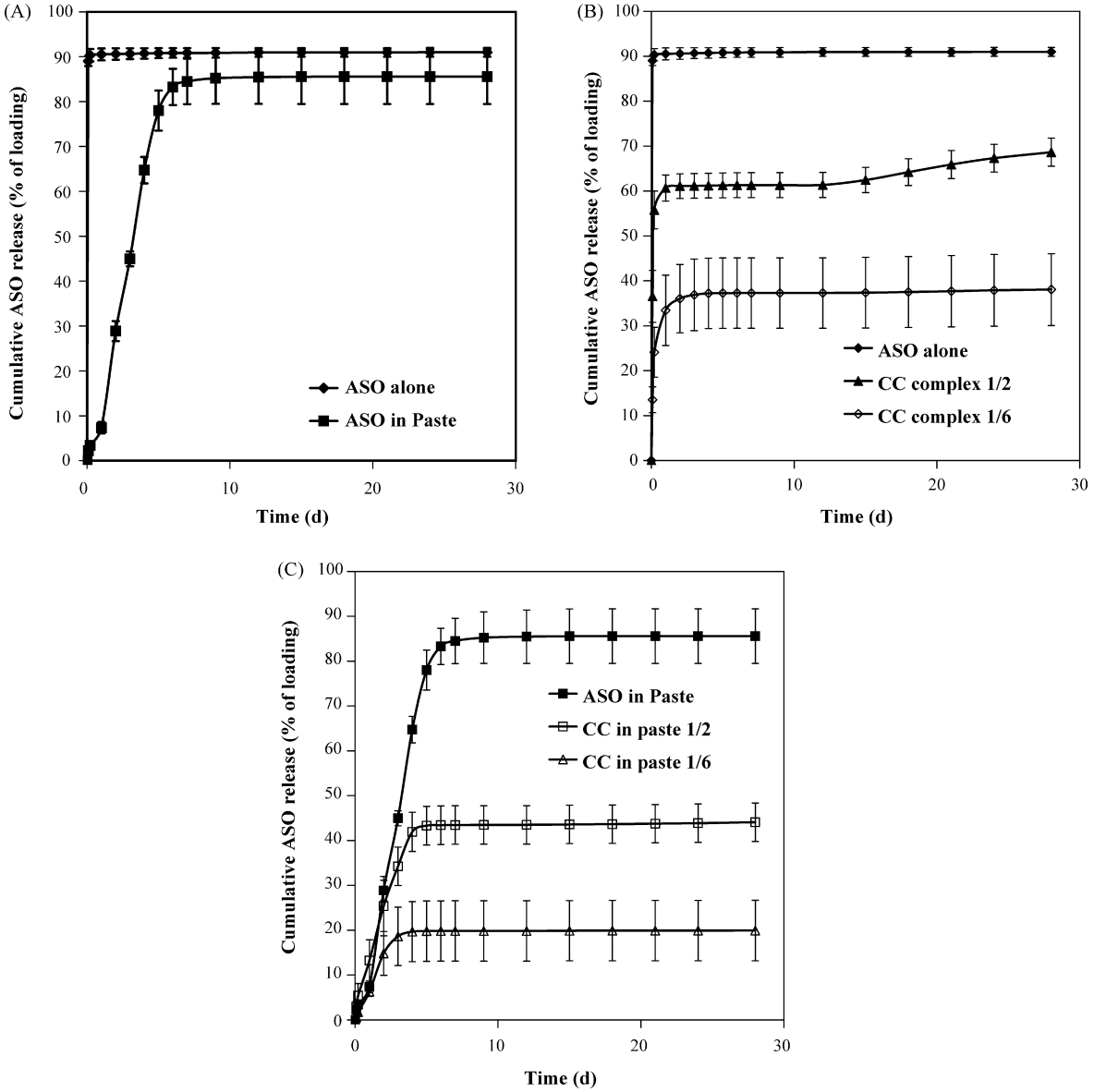

Fig. 3. Effect of clusterin antisense oligonucleotide (ASO)/chitosan ratio on the *in vitro* release profiles of clusterin ASO from various formulations incubated in phosphate buffered saline with sodium phosphate concentration of 10 mmol/L (pH 7.4) at 37 °C and analyzed by anion exchange high pressure liquid chromatography. Each formulation was loaded with 1 mg clusterin ASO. (A) Triblock copolymer/methoxy-poly(ethylene glycol) 40/60 w/w (polymeric paste). (B) Clusterin ASO/chitosan microparticle complexes (CC complexes). (C) CC complexes loaded into polymeric paste (CC in paste). Data points and error bars represent the mean ± standard deviation ($n = 5$).

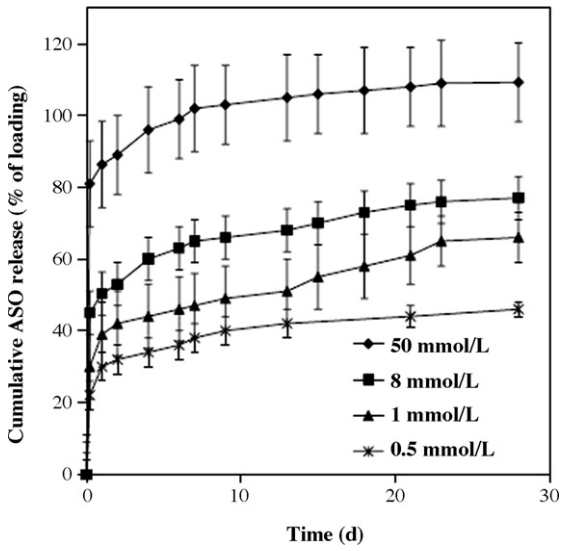

Fig. 4. Effect of sodium phosphate concentration on the *in vitro* release profiles of clusterin antisense oligonucleotide (ASO) from clusterin ASO/chitosan microparticle complexes (CC complexes) loaded into triblock copolymer/methoxy-poly(ethylene glycol) 40/60 w/w polymeric paste (CC in paste) incubated in phosphate buffered saline with differing sodium phosphate concentrations (pH 7.4) at 37 °C and analyzed by anion exchange high pressure liquid chromatography. Each CC in paste had a clusterin ASO/chitosan ratio of 1/2. The concentration of sodium phosphate is given for each release profile in the legend. Data points and error bars represent the mean ± standard deviation ($n = 4$).

day study, averaging between 0.1 and 0.3 μg per day (Fig. 3C). The rate and extent of clusterin ASO release increased as the sodium phosphate concentration was increased in the release media and the Clusterin ASO released rapidly from CC in pastes for the first 1–2 days, followed by a slower release for the remainder of the 28 day study (Fig. 4). The total amount of clusterin ASO released increased from 46% to approximately 100% as the sodium phosphate concentration was increased from 0.5 to 50 mmol L^{-1}.

3.7. *In vitro release profiles in human plasma*

Release profiles for full-length clusterin ASO from polymeric paste into PBS and plasma were similar (Figs. 3A and 5A). After incubation alone in plasma for 1 day, 88 ± 12% of the clusterin ASO was recovered as the sum of amounts of the 21-mer clusterin ASO and the 20-, 19- and 18-mer degradation products. The 21-mer clusterin ASO released from polymeric paste in a controlled manner over the first 7 days, followed by a slower release averaging 0.3 μg/day from day 7 to day 10, after which no oligonucleotides were detected in the plasma for the remaining 21 days (Fig. 5A). The 21-mer clusterin ASO released from CC complexes in a controlled manner for the first 3–4 days, followed by a slower release averaging 3–4 μg/day for the next several days until day 7 (Fig. 5B). No oligonucleotides were detected in the plasma from days 7 through 28. For the CC complexes at the end of the study, 62–64% of the clusterin ASO was accounted for as the sum of amounts of the 21-mer clusterin ASO and 20-, 19- and 18-mer degradation products, which was lower than the recovery from polymeric paste and CC in paste formulations, which had a total recovery of 91 to approximately 100% (Table 1). The 21-mer clusterin ASO released from CC in pastes in a controlled manner over the first 7 days, after which no oligonucleotides were detected in the plasma for the remainder of the study (Fig. 5C). After 28 days, CC in pastes had residual 21-mer clusterin ASO amounts of 20 and 44% for clusterin ASO/chitosan ratios of 1/2 and 1/6, while polymeric paste (no chitosan) and CC complexes had residuals of zero and 1–2% (Table 1).

3.8. *Western blots*

A representative set of Western blots for clusterin protein and vinculin protein (house-keeping protein) is provided in Fig. 6A and demonstrates the inhibition of the expression of the unprocessed 60 kDa form of clusterin protein following treatment with clusterin ASO alone or clusterin ASO released from the various formulations. Fig. 6B shows the relative clusterin expression following densitometry and standardization of clusterin expression of the no treatment group to 100%. Treatment with clusterin ASO alone and clusterin ASO released from the various formulations resulted in 52–62% inhibition of the expression of clusterin protein, which was significantly greater than the 22% inhibition of expression that resulted from treatment with mismatch control oligonucleotide ($p < 0.05$, ANOVA). There was no difference in clusterin expression between treatments with clusterin ASO alone and clusterin ASO released from the various formulations.

3.9. *In vitro degradation of clusterin ASO*

The stability of full-length 21-mer clusterin ASO either alone or complexed with chitosan at a w/w ratio of 1/6 was determined in 50% human plasma incubated at 37 °C for 4 days. In addition, the amounts of the 20-, 19- and 18-mer degradation products were determined. At the end of 4 days approximately 40% of the full-length clusterin ASO remained in both the clusterin ASO (no chitosan) and the CC complex 1/6 samples. At the end of 4 days an amount of 20-mer degradation oligonucleotide equivalent to approximately 20% of the initial 21-mer clusterin ASO was determined to be present in both the clusterin ASO (no chitosan) and the CC complex 1/6 samples.

4. Discussion

The successful outcome of intratumoral targeted ASO delivery is dependent on the development of delivery systems that are able to release bioactive ASOs within a solid tumor in a sustained manner. We previously described an injectable, intratumoral delivery system comprising polyanionic clusterin ASO loaded into polycationic chitosan microparticles (CC complexes) incorporated in a biocompatible and biodegradable polymeric paste (CC in paste). The system was found to provide controlled release of clusterin ASO *in vitro* (Springate et al., 2005). When paclitaxel or docetaxel was also blended into the CC in paste, intratumoral injection of these formulations into human prostate

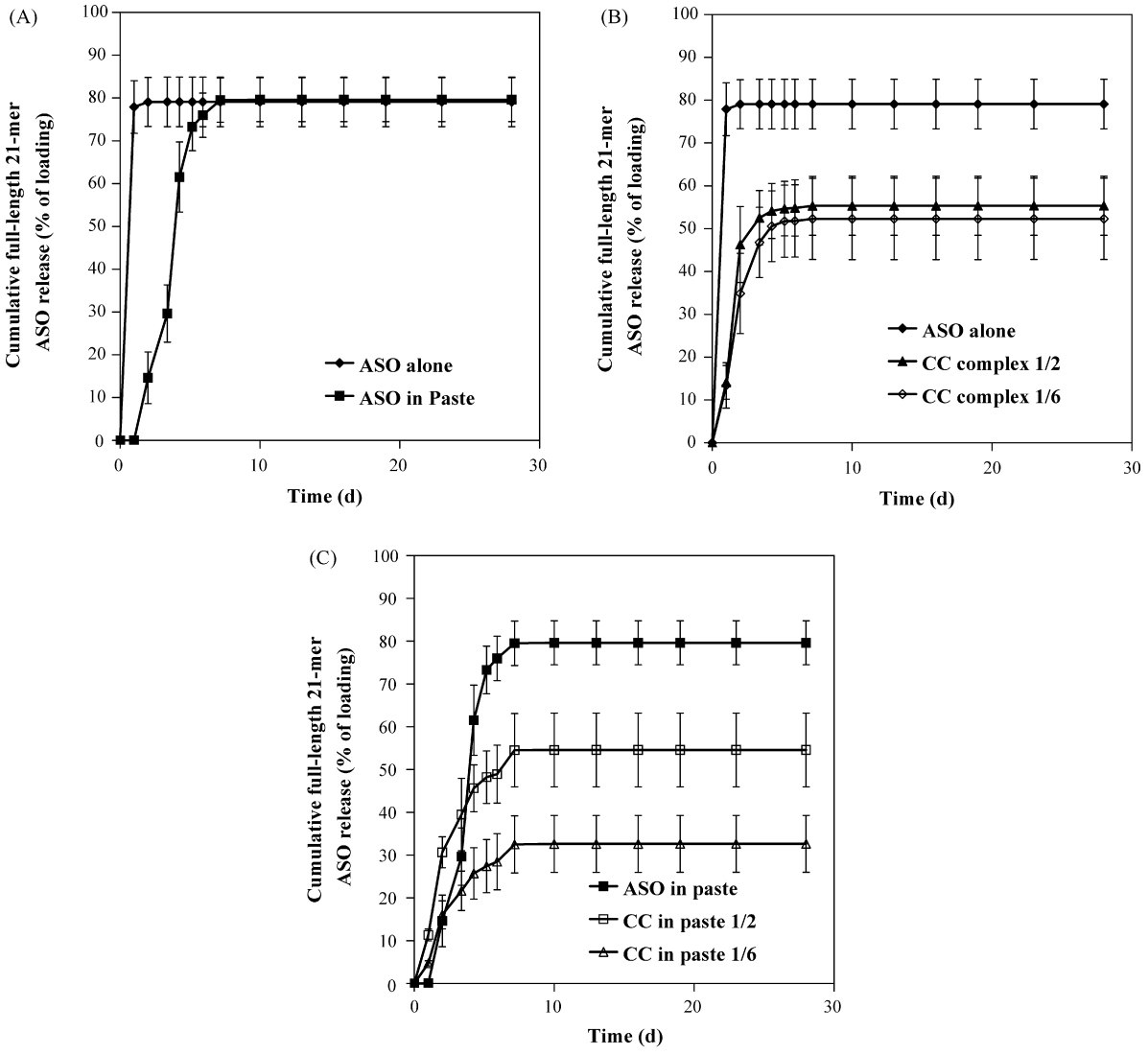

Fig. 5. Effect of clusterin antisense oligonucleotide (ASO)/chitosan ratio on the *in vitro* release profiles of full-length 21-mer clusterin ASO from various formulations incubated in human plasma at 37 °C and analyzed by capillary gel electrophoresis. Each formulation was loaded with 1 mg clusterin ASO. (A) Triblock copolymer/methoxy-poly(ethylene glycol) 40/60 w/w (polymeric paste). (B) Clusterin ASO/chitosan microparticle complexes (CC complexes). (C) CC complexes loaded into polymeric paste (CC in paste). Data points and error bars represent the mean ± standard deviation ($n = 5$).

Table 1
Cumulative *in vitro* release and residual analysis of full-length 21-mer clusterin antisense oligonucleotide and 20-, 19- and 18-mer catabolites from various formulations incubated in human plasma at 37 °C and analyzed by capillary gel electrophoresis

Formulation name	Cumulative 21-mer ASO released by 28 days (% of ASO loaded)	Residual 21-mer ASO in each pellet[a] at 28 days (% of ASO loaded)	Total 21-mer ASO released + in each pellet at 28 days[b] (% of ASO loaded)	Cumulative release of 20-, 19- and 18-mer oligos (% of ASO loaded)	Residual 20-, 19-, and 18-mer oligos at 28 days[c] (% of ASO loaded)	Total 21-mer ASO and 20-, 19- and 18-mer oligos released + in each pellet at 28 days[d] (% of ASO loaded)
ASO alone[e]	79	n/a	79 ± 6	9	n/a	88 ± 12
ASO in paste	80	0	80 ± 5	14	0	93 ± 9
CC complex 1:2	55	1	57 ± 7	6	1	64 ± 8
CC complex 1:6	52	2	55 ± 10	6	1	62 ± 12
CC paste 1:2	55	20	75 ± 9	8	3	86 ± 19
CC paste 1:6	33	44	76 ± 8	10	5	91 ± 13

[a] Amount of full-length 21-mer clusterin ASO remaining in the semi-solid pellet at the end of the release study.
[b] Sum of the cumulative release and the amount of full-length 21-mer clusterin ASO remaining in the semi-solid pellet at the end of the release study.
[c] Sum of the amounts of 20-, 19- and 18-mer degradation products remaining in the semi-solid pellet at the end of the release study.
[d] Sum of the cumulative release and the amounts of 21-mer full-length clusterin ASO plus 20-, 19- and 18-mer degradation products in the semi-solid pellet at the end of the release study.
[e] Clusterin ASO alone (no formulation) was incubated under the same conditions as the various formulations.

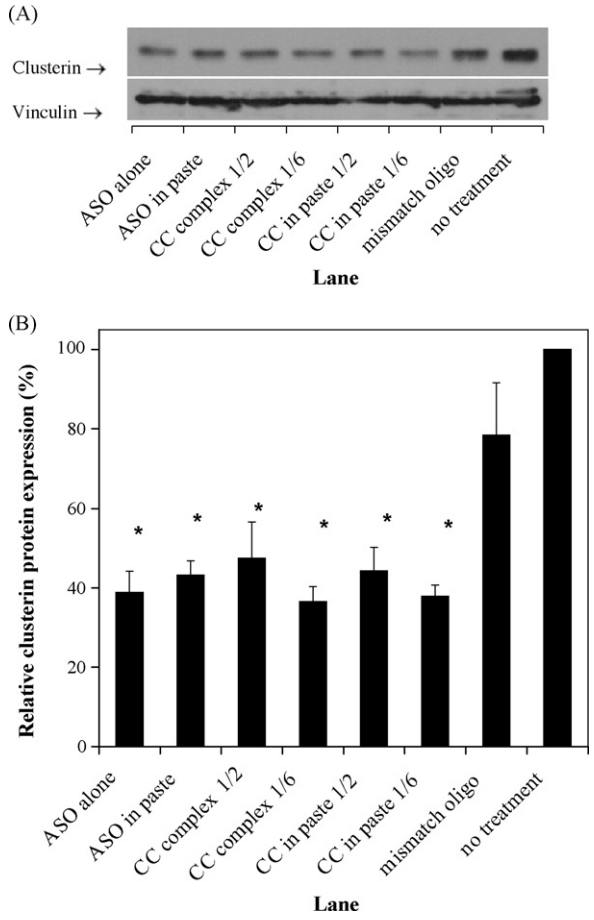

Fig. 6. Effect of clusterin ASO/chitosan microparticle complexes (CC complexes), triblock copolymer/methoxy-poly(ethylene glycol) 40/60 w/w (polymeric paste), and CC complexes loaded into polymeric paste (CC in paste), on the *in vitro* bioactivity of clusterin ASO. Clusterin ASO released from the various formulations into phosphate buffered saline was analyzed by high pressure liquid chromatography and the clusterin ASO concentration standardized to 500 nmol/L before treating PC-3 cells. (A) Western blots of clusterin protein and vinculin protein (vinculin was used as a housekeeping protein) in PC-3 cells. (B) Densitometry of Western blots' bands. Clusterin protein expression was divided by vinculin protein expression and reported in the figure as a percent of clusterin protein expression by the PC-3 cells treated with control (no oligonucleotide) standardized to 100%. (*) Signifies significant difference in clusterin protein from mismatch control oligonucleotide treatment (ANOVA, $p < 0.05$).

cancer tumors in mice produced *in vivo* efficacy over 4 weeks (Springate et al., 2005). In the current studies, the effect of clusterin ASO/chitosan ratio on the physicochemical properties of CC complexes and the influence of chitosan and polymeric paste on the *in vitro* release and stability of clusterin ASO were investigated.

Chitosan is a biocompatible material with an established history of use as a plasmid and antisense oligonucleotide complexation and cell transfection agent (Renbutsu et al., 2005; Gao et al., 2005). The amine group on the *N*-D-glucosamine residue is capable of accepting a proton in acidic solutions and the potential maximum number of positive charges on chitosan is limited by the number of *N*-D-glucosamine monomers. For plasmid transfection either water soluble chitosan derivatives or acidic solutions of chitosan were used to condense DNA into nanoparticulate suspensions (Ramos et al., 2005). In this study, insoluble microparticulate chitosan was found to swell by approximately 600% in aqueous media at approximately neutral pH so that amine groups within the chitosan hydrogel matrix might be exposed to water without dissolution to bind ASO and provide a complex that might be used for controlled release purposes. The strength of the binding between the polyanionic clusterin ASO and the polycationic chitosan depends on the degree of protonation (positive charge) of the amine groups on the polysaccharide chains (Romoren et al., 2003) which is influenced by the degree of deacetylation of the chitosan and the pH of the media. The effect of pH on the charge on the chitosan chains may best be described by the pK_a which reports the pH at which 50% of the amine groups are protonated. Using chitosan with a degree of deacetylation of approximately 85%, the pK_a value of the polysaccharide was found to lie between 6.2 and 6.7 at ionic strengths of 0 and 0.1, respectively. Similarly, others showed that the pK_a of high MW chitosan ranged from 5.5 to 7.1 as the degree of deacetylation was decreased from 95 to 78% while the ionic strength of the media was increased from 0.01 to 0.1 (Romoren et al., 2003; Peng et al., 2005). The chitosan microparticles swelled in PBS and CC complexes had mean diameters of approximately 200 μm (much larger than coacervate or condensation plasmid/chitosan used for transfection (Ishii et al., 2001)) which may provide a depot or implant type of formulation following intratumoral injection, compared to colloidal or coacervate nanoparticulate complexes (Springate et al., 2005).

Clusterin ASO was found to bind to microparticulate chitosan so that all of the clusterin ASO in solution could be fully bound and removed from solution by increasing the chitosan content of CC complexes to a clusterin ASO/chitosan ratio of 1/4 or higher (Fig. 2). This binding was pH dependent so that as the pH dropped from 7.4 to 7.0 and 6.6, the number of positive charges in the chitosan microparticles increased and more clusterin ASO could be bound for a given amount of chitosan. Similar results of plasmid/chitosan condensation complexes were reported by others (Lee et al., 2001; Köping-Höggård et al., 2001; Romoren et al., 2003). These effects of weight ratios and pH on the net charge of the microparticles were further supported by zeta potential determinations. The microparticulate charge increased from approximately −10 mV to positive values as chitosan content of CC complexes increased with greater rates of change occurring as the pH dropped from 7.4 to 7.0 and 6.6 (Fig. 1). As the amount of chitosan was increased relative to clusterin ASO or as the pH was decreased, the number of protonated chitosan amines increased, resulting in less negatively charged or more positively charged CC complexes and a corresponding change in zeta potential values for the microparticles in agreement with similar studies by other workers (Erbacher et al., 1998). The microparticulate CC complexes are difficult to suspend in aqueous media or to inject through syringe/needle assemblies due to sedimentation or aggregation during injection. However, when CC complexes were incorporated into a polymeric paste consisting of a 40/60 blend of triblock copolymer and MePEG, sedimentation and aggregation issues were overcome and the formulation was easily injected through a 22 gauge needle.

Clusterin ASO released from polymeric paste, CC complexes and CC in pastes *in vitro* in PBS in a bioactive form (Fig. 6), suggesting that the clusterin ASO was released from the various formulations as "free" ASO (not complexed with chitosan). When clusterin ASO was suspended in polymeric paste in the uncomplexed form, all the oligonucleotide released in 1 week (Figs. 3A and 5A). This release over a short time period may have resulted from the uniform, hemispherical geometry of the paste pellets which may have determined the rate of entry of water into the core and subsequent release of the ASO. The uncomplexed ASO in paste (no chitosan) system would have little practical application *in vivo* where the geometry of the paste would be highly variable following intratumoral injection. Clusterin ASO released from CC in pastes *in vitro* in both PBS and plasma (Figs. 3C and 5C) in a sustained manner over the first 4–7 days, followed by no or very little release for the remainder of the study. When plotted against the square root of time the curves became linear suggesting that ASO was released from polymeric paste formulations in the presence or absence of chitosan, at least in part, via diffusion controlled release. However, clusterin ASO released from CC complexes *in vitro* in both PBS and plasma in a rapid manner over the first 1–4 days, followed by a slower release phase for the next several days (Figs. 3B and 5B). These non-paste release profiles showed no linear relationship (data not shown), suggesting that ASO was released from chitosan complexes by a different mechanism than simply diffusion alone. The rate and extent of clusterin ASO release from CC in pastes in PBS increased as the sodium phosphate concentration was increased in the release media (Fig. 4). Phosphate ions may compete with or shield the polyanionic clusterin ASO chains from positively charged binding sites on the chitosan (similar to the HPLC analysis of ASO using a gradient of increasing perchlorate ions as these ions (like phosphate ions) compete with the clusterin ASO for sites on the column, thereby displacing (or "releasing") the ASO). This competitive ion effect supports the zeta potential, complexation, and release data which point towards clusterin ASO interacting with chitosan microparticles through electrostatic bonds to form microparticulate CC complexes (Mao et al., 2001; Peng et al., 2005).

Modifying oligonucleotides by replacing one of the non-bridging oxygen atoms on each of the inter-nucleoside phosphate groups with a sulphur atom (termed fully phosphorothioated oligonucleotide) provides some resistance to nuclease degradation (Gilar et al., 1997). However, due to their short tissue half-lives, phosphorothioated ASOs are administered in the clinical setting by continuous or frequent infusion (O'Brien et al., 2005). Clusterin ASO was formulated in CC in paste, in part, to improve the stability of the ASO. At the termination of the release studies after 4 weeks, totals of cumulative release plus residual clusterin ASO in PBS for all the formulations evaluated, accounted for approximately 80–100% of the loaded clusterin ASO. However, similar determinations in human plasma accounted for approximately 80% of the full-length 21-mer clusterin ASO in CC in pastes and only approximately 55% of the full-length 21-mer clusterin ASO in CC complexes without polymeric paste (Table 1). We suggest that CC in paste formulations may have protected the clusterin ASO from degradation by nucleases present in plasma. We also suggest that in the case of CC complexes without paste, nucleases present in plasma (but not present in PBS) penetrated the swollen chitosan hydrogel matrix and degraded the clusterin ASO, leaving only 1–2% of the full-length 21-mer clusterin ASO in the CC complexes at the end of the study (Table 1). The degradation study showed no difference in the degradation profiles of full-length 21-mer clusterin ASO when incubated in 50% human plasma alone or complexed with chitosan at a ratio of 1/6. These data provide additional evidence that the ASO complexed to chitosan was not protected from degradation by nucleases in plasma. However, in the case of CC in pastes, the penetration of nucleases into the CC complexes was inhibited by the semi-solid/waxy triblock copolymer, leaving 20–44% of the full-length 21-mer clusterin ASO in the CC in pastes at the end of the release study in plasma (Table 1).

A schematic of a proposed mechanism of release of clusterin ASO from CC in paste *in vitro* is shown in Fig. 7. Initial incubation of the CC in paste in an aqueous media results in water diffusing into the paste (see pathway 1). The water soluble MePEG diffuses out of the paste, leaving interconnected aqueous pores in the remaining semi-solid triblock copolymer (see pathway 2), allowing water to swell the chitosan microparticles (see pathway 3). As clusterin ASO is released from the CC complexes, free clusterin ASO diffuses through the aqueous pores and channels in the triblock copolymer and is released (see pathway 4). After approximately 1 week most of the clusterin ASO remaining in the chitosan particles is strongly bound to

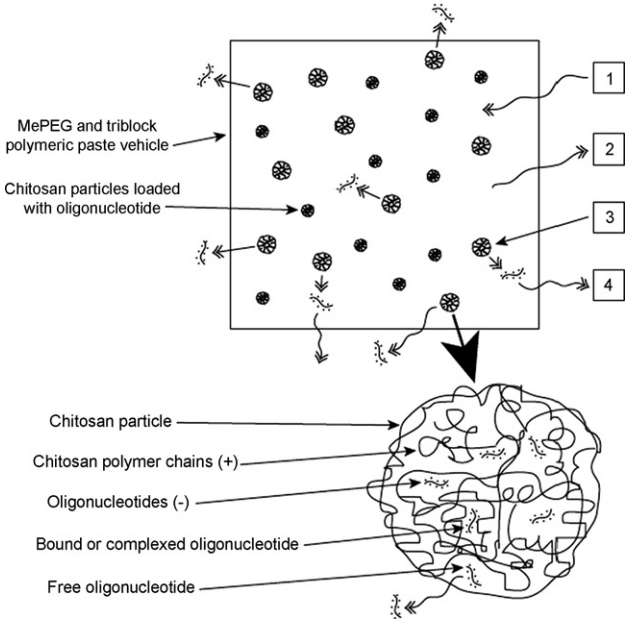

Fig. 7. Schematic illustrating the proposed mechanism of antisense oligonucleotide (ASO) release following complexation with chitosan microparticles (CC complexes) and loading of CC complexes into triblock copolymer/methoxy-poly(ethylene glycol) (MePEG) 40/60 w/w (polymeric paste) (CC in paste). ASO release occurs as follows: (1) Water diffuses into the MePEG and polymeric paste vehicle. (2) Water-soluble MePEG diffuses out of the polymeric paste, resulting in the formation of pores and channels in the remaining triblock paste. (3) Water swells chitosan particles. (4) "Free" oligonucleotide diffuses through pores and channels out of the polymeric paste.

the chitosan through electrostatic complexes and is very slowly released from CC complexes *in vitro* over the next 3 weeks. We previously showed that a single intratumoral injection of CC in paste with paclitaxel or docetaxel incorporated into the paste resulted in efficacy for 4 weeks against PC-3 and LNCaP human prostate tumors grown in mice (Springate et al., 2005). We speculate it is possible that the presence of numerous salt ions *in vivo* may play a role as counter ions in the release of clusterin ASO *in vivo* (Köping-Höggård, 2003) and that chitosan is also likely undergoing degradation *in vivo* due to enzymes such as lysozyme, which may assist the *in vivo* release of ASO from the CC complexes (Mi et al., 2002; Freier et al., 2005). From the *in vitro* clusterin ASO release data presented in this work and *in vivo* efficacy data presented earlier (Springate et al., 2005), we suggest that clusterin ASO is likely released *in vivo* from CC in pastes in a sustained manner over approximately 4 weeks.

5. Conclusions

Clusterin ASO/chitosan ratio and pH of the environment both influenced the electrostatic interaction of clusterin ASO with chitosan in CC complexes. The rate and extent of clusterin ASO release was related to the clusterin ASO/chitosan ratio and to whether the clusterin ASO or CC complexes were incorporated into polymeric paste (CC in paste). Polymeric paste protected clusterin ASO from degradation by nucleases *in vitro*. Clusterin ASO released from the various formulations suppressed the expression of clusterin protein *in vitro*.

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