Surface-Modified Nanoparticles Enhance Transurothelial Penetration and Delivery of Survivin siRNA in Treating Bladder Cancer

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Abstract

Penetration of the bladder permeability barrier (BPB) is a major challenge when treating bladder diseases via intravesical delivery. To increase transurothelial migration and tissue and tumor cell uptake, poly(lactic-co-glycolic acid; PLGA) nanoparticles (NP) were modified by addition of a low molecular weight (2.5 or 20 kDa) positively charged mucoadhesive polysaccharide, chitosan, to the NP surface. In designing these NPs, we balanced the adhesive properties of chitosan with the release and bioactivity of the siRNA. Chitosan-functionalized NPs demonstrated increased binding to and uptake in intravesically instilled mouse bladders and human ureter at 10 times the level of unmodified NPs. Furthermore, we extended the bioactivity of survivin siRNA in vitro for up to 9 days and demonstrated a decrease in proliferation when using chitosan-modified NPs relative to unmodified NPs. In addition, treatment of xenograft tumors with chitosan-modified NPs that encapsulate survivin siRNA (NP-siSUR-CH2.5) resulted in a 65% reduction in tumor volume and a 75% decrease in survivin expression relative to tumors treated with blank chitosan NPs (NP-Bk-CH2.5). Our low molecular weight chitosan delivery system has the capacity to transport large amounts of siRNA across the urothelium and/or to the tumor site, thus increasing therapeutic response.

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Introduction

Oral or systemic administration of drugs for the treatment of pathologic conditions affecting the bladder is often only partially effective because a limited portion of drug reaches the affected site. The remainder of the drug is distributed to other tissues, decreasing efficacy and resulting in adverse side effects (1, 2). Localized, intravesical instillation of therapeutic agents avoids first-pass metabolism associated with systemic and oral delivery, allowing the drugs/siRNAs to be delivered more efficiently and at higher dosage (3), thus minimizing adverse effects. However, the challenges of localized delivery of drugs and siRNA to the bladder include low urothelium penetration, short half-life, increased dilutions (3, 4), and short retention times (5). To circumvent the preceding caveats of freely delivered drug/siRNA, we encapsulated siRNA in poly(lactic-co-glycolic acid; PLGA) nanoparticles (NP) for bladder administration (6).

PLGA is a biocompatible, degradable polymer approved by the U.S. Food and Drug Administration (FDA) that has been used to encapsulate and deliver drugs, siRNAs, DNAs, peptides, and proteins (7–11). PLGA NPs can protect biologic agents from degradation during systemic circulation (12), thereby extending drug efficacy. Our group previously has used the solvent emulsion method (water/oil/water) to successfully and efficiently entrap a variety of agents. Our NPs can efficiently encapsulate siRNAs (9) and drugs (13), provide controlled release of biologic agents (8), and can be modified for targeted delivery (14). The main focus of this study is to improve the application of these NPs as a more effective delivery modality.

The bladder permeability barrier (BPB) or urothelium is an effective biomembrane barrier, which consists of umbrella cells, tight junctions, and plaques (1). Although the BPB is impermeable to most substances, modifications to the urothelial cells or tight junctions can increase barrier permeability (1, 15), which is critical for drug/siRNA penetration through the bladder wall. Uptake of drugs/siRNAs can be improved by modifying NPs to enhance binding and internalization. Surface modifications with natural polysaccharides and cell-penetrating peptides (CPP) are known to provide increased cell adhesion, internalization, and tissue penetration (1). Penetratin (AP) is derived from the Drosophila melanogaster transcription factor of the antennapedia homeodomain and has been...
used to enhance intracellular delivery (16). AP is an amphipathic CPP with short cationic sequences that can cross the cell membrane through a receptor-independent mechanism (16–18). In comparison, chitosan (CH) is a biocompatible, nontoxic, and mucoadhesive polysaccharide, derived from the exoskeletons of crustaceans, with an established safety profile in humans (3, 19, 20). Chitosan can adhere to bladder urothelium, and its high viscosity may make it more resistant to excretion. Previously, our group demonstrated the feasibility of using avidin as an adaptor protein to enable the attachment of biotinylated ligands to the surface of NPs (14). In this study, avidin-modified PLGA NPs were conjugated with biotinylated AP or CH, to facilitate enhanced cell adhesion and internalization and to increase NP transurothelial migration and tumor uptake in urothelial cells and tissue.

In this study, we modified PLGA NP surfaces with AP, CH 2.5 kDa (CH2.5), and CH 20 kDa (CH20). By varying the CH on the NPs, we tested for the effect of CH chain length on binding, internalization, and/or release of encapsulated molecules from the NPs. To evaluate NP binding and internalization in encapsulated molecules from the NPs. To evaluate NP length on binding, internalization, and/or release of CH 2.5 kDa (CH2.5), and CH 20 kDa (CH20). By varying tissue.

isoRNA is effective in reducing bladder tumor growth in a mouse bladder, NPs were loaded with a hydrophobic, green fluorescent dye, coumarin-6 (C6). C6 is encapsulated within our NPs to track the NPs within cells and tissue. NP uptake was evaluated by measuring total fluorescence in vivo in intravesically instilled mouse bladders, as well as in human nonneoplastic diluted ureters. We qualitatively determined NP uptake via fluorescence microscopy, and quantified binding and internalization using fluorescence-activated cell sorting (FACS). We also tested the ability of these NPs to deliver survivin siRNA. Survivin, an inhibitor of apoptosis, is highly expressed in bladder cancer cells (21, 22). We assessed siRNA release from surface-modified NPs in artificial urine and evaluated its effect on survivin mRNA expression in vitro and in vivo.

We hypothesize that the design of a drug-delivery vehicle, such as surface-modified NPs, will enhance drug uptake, increase therapeutic residence time, and increase the solubility of drugs, thereby enhancing efficacy. For the first time, we show that administration of low molecular weight CH-modified PLGA NPs encapsulating survivin siRNA is effective in reducing bladder tumor growth in a xenograft murine model.

Materials and Methods

Formulation and characterization of surface-modified NPs

C6 NP fabrication. C6-loaded NPs were fabricated with the following surface modifications: (i) unmodified (NP-C6-Unmod), (ii) avidin-only-modified (NP-C6-AP), (iii) AP-modified (NP-C6-AP), (iv) 2.5 kDa chitosan–modified (NP-C6-CH2.5), and (v) 20 kDa chitosan–modified (NP-C6-CH20). Peptides were synthesized by the W.M. Keck Facility at Yale University, and contained a Ser-Gly dipeptide spacer between the N-terminal amino acid and biotin.

Before NP synthesis, avidin-palmitate was conjugated to the surface of PLGA NPs (50:50 carboxy-terminated; inherent viscosity range, 0.55–0.75 dL/g; LACTEL) as previously described (14). Briefly, the avidin-palmitate was prepared by reacting 10 mg of avidin with 14-fold molar excess of palmitic acid-N-hydroxysuccinimide ester in 2% sodium deoxycholate in PBS (37°C, 12 hours), followed by overnight dialysis.

C6 NPs were synthesized using a single oil-in-water emulsion technique (14, 23). One hundred milligram PLGA was dissolved overnight in 1 mL methylene chloride (DCM; oil). C6 (Acros Organics) was added to the DCM polymer solution (15 mg C6/mg polymer), vortexed, and sonicated. For unmodified NPs, the PLGA-C6 solution was added dropwise to 2.5% polyvinyl alcohol (PVA) alone (aqueous), whereas for modified NPs, the PLGA-C6 solution was added to 2.5% PVA containing 1 mg/mL avidin-palmitate. Both solutions were vortexed and sonicated. After the sonication/emulsion step, the emulsified solution was added to a 0.3% PVA mixture while stirring to evaporate the DCM. After 3 hours, this solution (DCM evaporated, leaving NPs in PVA) was added to centrifuge tubes and centrifuged/washed three times with deionized water to remove the PVA. After 3 washes/centrifuges, the NPs were suspended in 5 to 10 mL of deionized water, frozen at −80°C, and subsequently lyophilized to produce dry NPs.

After NPs were hardened during solvent evaporation, those with surface modification were reacted with 10 times molar excess biotin-CH2.5, CH20, or AP to avidin in PBS for 30 minutes. Unmodified NPs were incubated in PBS for 30 minutes without ligand. After the reaction, NPs were washed twice in deionized water to remove residual solvent, centrifuged at 4°C, lyophilized, and stored at −20°C until use.

Survivin siRNA NP fabrication. siRNA NPs were synthesized using the double emulsion solvent evaporation technique (water/oil/water) as this method is commonly used to prepare biodegradable hydrophobic NPs containing hydrophilic cargo (9). Briefly, 100 mg PLGA were dissolved overnight in 1 mL DCM (oil). The following day, complexes were formed between the siRNA and spermidine with an 8:1 molar ratio of the polyamine nitrogen to the nucleotide phosphate (N:P). The survivin target sequence (siSurvivin) was 5′-AAGGACCACCGCAUCUCUACA-3′ (sense), with the scramble control: 5′-AACGUAACCGGAUACUUCGA-3′ (Dharmacon). siRNA–spermidine complexes were incubated at room temperature for 15 minutes before NP synthesis. One hundred nanomoles of siRNA per 100 mg polymer in Tris-EDTA (10 mmol/L Tris-HCl, 1 mmol/L EDTA) buffer (aqueous) was added dropwise to the PLGA solution while vortexing. This solution was sonicated and subsequently added to a 2.5% PVA solution (with or without avidin depending on surface modification; aqueous) for the second emulsion. NPs were hardened during solvent...
evaporation in 0.3% PVA for 3 hours. The hardened NPs were washed three times with deionized water to remove residual solvent, centrifuged at 4°C, lyophilized, and stored at −20°C until use.

**NP characterization: siRNA loading, encapsulation efficiency, and size**

To determine siRNA loading and encapsulation efficiency, NPs were incubated with DCM, and siRNA was extracted into aqueous buffer as described previously (9). Briefly, 3 to 5 mg of siRNA NPs were dissolved in 0.5 mL of DCM for 30 minutes, and the siRNA was extracted into Tris-EDTA buffer. The quantity of extracted double-stranded siRNA was determined using the Quant-iT PicoGreen assay (Invitrogen). Fluorescence was compared with a known siRNA standard. The unmodified NPs encapsulated 600 pmol siRNA/mg NP, corresponding to an encapsulation efficiency of 60%. Our theoretical loading of the NPs was 1,000 pmol (1 nmol). Both formulations of surface-modified chitosan NPs encapsulated 700 pmol survivin siRNA/mg NP, with a corresponding encapsulation efficiency of 70%. NP morphology, diameter, and size distribution were determined from scanning electron microscope images using ImageJ.

**Fluorescence-activated cell sorting**

T-24 and UM-UC-3 bladder cancer cells were obtained directly from the American Type Culture Collection in 2007 and 2010, respectively. The cells were authenticated and underwent quality control using short tandem repeat DNA profiles, morphology verification, growth assays, and mycoplasma testing during these experiments. In addition, only low-passage cells were used. UM-UC-3 and T-24 cells were maintained in Eagles Minimum Essential and McCoy’s medium, respectively. Both cell lines were supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% glutamine. UM-UC-3 or T-24 cells were incubated with NP-C6-AV, NP-C6-AP, NP-C6-CH2.5, and NP-C6-CH20 at 1 mg/mL for 2 hours in a 37°C humidified chamber, in the appropriate medium. The avidin control group represents NPs with no CPP (or targeting ligand), as we wanted to ensure that the avidin modification was not the reason for enhanced binding/targeting ligand, as we wanted to ensure that the avidin modification was not the reason for enhanced binding/uptake. Then, ice-cold medium was added to stop endocytosis. Cells were washed with PBS, detached with trypsin-EDTA, and either fixed in FACS solution containing 1% (v/v) of FBS and paraformaldehyde (PFA) in PBS or exposed to 0.3% trypsin blue for 2 minutes before being fixed in FACS solution (24, 25). Trypan blue quenches extracellular surface fluorescence of living cells, allowing for quantification of internalized fluorescence (25). The cells then were sorted using a BD LSR II, located in the Cell Sorter Facility at Yale University. Data were analyzed using FlowJo software, and a minimum of 20,000 cell-events were analyzed per sample. To overlay histograms containing different number of events collected, the FlowJo software would use percentage of maximum (% Max) for normalization. The % Max is the number of cells in each bin divided by the number of cells in the bin that contains the greatest number of cells (26).

**Fluorescence microscopy**

UM-UC-3 or T-24 cells were grown on coverslips for 48 hours until 80% to 90% confluence. Cells were incubated with NP-C6-AV, NP-C6-AP, NP-C6-CH2.5, and NP-C6-CH20 at 1 mg/mL for 2 hours at 37°C in a CO2 incubator. The cells were incubated with or without 0.3% trypsin blue, washed in PBS, and fixed with 3.7% PFA. The cells were then incubated with 0.1% Triton X-100 before being treated with Texas Red phalloidin (Invitrogen), to visualize the F-actin. The coverslips were mounted on slides with VECTASHIELD containing 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei.

**Ex vivo human ureter system**

The use of biologic specimens for human research was approved by the Human Investigation Committee at Yale University (Protocol number, 0710003157). The uptake of surface-modified NPs was measured in an in vivo binding assay, using human nonneoplastic dilated ureters. The ureters were used within 4 hours of removal. After removing external connective tissue and washing in sterile PBS, the ureter was placed in an autoclaved 96-well dot blot chamber (Bio-Rad) with the luminal urothelium facing upward. Unmodified and surface-modified NPs, loaded with C6 (100 µL of 2 mg/mL), were suspended in artificial urine and added to individual wells. Artificial urine alone was used as a control. The apparatus was incubated at 37°C in a humidified incubator for up to 2 hours. After incubation, wells were washed four times to remove nonadherent NPs, cored with a biopsy punch, and weighed. The samples were frozen for extraction of C6.

**In vivo mouse intravesical instillation**

All animal studies were approved by the Institutional Animal Care and Use Committee of Yale University. Female mice were intraperitoneally sedated with ketamine (100 mg/kg) and xylazine (10 mg/kg). External genitalia were cleansed. Then, the mice were catheterized with a lubricated 24G catheter (BD Biosciences), and their bladders were emptied. The catheter was then attached to a Hamilton syringe filled with 100 µL of artificial urine with or without surface-modified, C6-loaded NPs (2 mg/mL). The solution was instilled into the bladder, the catheter was removed, and an ultra-small clamp was placed on the external urethra (Natsume Seisakusho Co., Ltd), which remained in place for 2 hours, the typical amount of time used for clinical intravesical treatments. Mice then were sacrificed; bladders were washed extensively with PBS to remove nonadherent NPs and weighed. Samples were frozen for extraction of C6.

**Extraction of C6 from human and mouse tissue**

Distilled water (750 µL) was added to frozen human urethral tissue cores or mouse bladders and the tissue...
was homogenized on ice using a Polytron homogenizer (Brinkman). The homogenate was vortexed for 1 hour at room temperature using the VX-2500 Multi Tube Vortexer (VWR) at the highest speed (setting 10) and then centrifuged (16,000 × g; 10 minutes). After removal of the supernatant, dimethyl sulfoxide (DMSO; a solvent that dissolves the PLGA NPs) was added so that the weight (g)/volume (mL) ratio was 1:10. The samples were again vortexed and centrifuged. C6 fluorescence in the samples (100-μL aliquots) was measured on a 96-well plate reader at 460 nm excitation, 540 nm emission. Fluorescence in the DMSO supernatant was divided by total (input) fluorescence to determine percentage fluorescence or by mouse bladder or tissue weight.

### Quantitative real-time PCR

Total RNA was extracted from cells or murine tumors using TRIzol (Invitrogen). cDNA was synthesized using iScript (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad), 100 nmol/L specific primers and equivalent amounts of cDNA. Melting curves for all primers produced a single peak. For mRNA analysis, the relative level of gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, using the cycle threshold method. The following primers were used: GAPDH sense, 5'-CCACTAGGCGCTACGTCTTCT-3'; antisense, 5'-GGGACTACCGCGGTGTTAGTCT-3'; survivin sense, 5'-GACCCAGTGTTCTCATGTC-3'; and anti-sense, 5'-TGCTTTTATGTGCTTTATGTTGG-3'.

### In vitro siRNA transfection and cell proliferation assay

UM-UC-3 cells were seeded in a six-well plate overnight until they reached a cell density of 20%. The cells were transfected with siSurvivin to a final concentration of 100 nmol/L with Lipofectamine RNAimax (Invitrogen). After 3 and 9 days at 37°C in a 5% CO2 incubator, the cells were harvested to determine gene expression. For the 9-day siSurvivin study, UM-UC-3 cells were trypsinized and replated with new media after the fourth day. Also, on day 9, a cell proliferation assay was performed in which bladder cancer cells that were treated with NP-siSUR-Unmod, NP-siSUR-AV, NP-siSUR-CH2.5, or NP-siSUR-CH20 were trypsinized and counted in a TC10 automated cell counter (Bio-Rad).

### Controlled release of siRNA from NPs

NPs (4 mg) containing fluorescein-labeled siSurvivin (F-siSurvivin) were suspended in 1 mL of artificial urine and incubated at 37°C with gentle shaking over 13 days in a single conical tube. Cumulative release of siRNA was measured at predetermined time points. At each time point, the sample was centrifuged at 12,000 × g and the supernatant was collected. Once the supernatants were collected, the extracted F-siSurvivin was measured on a 96-well plate reader at 488 nm excitation, 520 nm emission wavelengths.

### Xenograft model

Eight-week-old female Foxn1 nu/nu mice were subcutaneously injected in the left flank with 5 × 10⁶ UM-UC-3 cells, which have high metastatic potential and are commonly used in xenograft models. The tumor volume was estimated using the formula (tumor length × tumor width²) × π/6. The mice were randomly divided into three groups: PBS (control), NP-Bk-CH2.5 (empty NPs), or NP-siSUR-CH2.5, when the tumor volumes reached 100 ± 15 mm³. Mice were intratumorally injected with: PBS, 1 mg/100 μL of NP-Bk-CH2.5 diluted in PBS or 0.7 mg/100 μL NP-siSUR-CH2.5 (500 pmol siSUR) diluted in PBS. Mouse tumors were intratumorally injected on day 0, 4, and 7, with day 0 being 4 days after inoculation of bladder cancer cells. Tumor volumes were measured before each injection. All animals were sacrificed on day 11 (15 days after cell inoculation). Xenograft mouse tumors were weighed, snap-frozen, and stored at −80°C.

### Statistical analysis

Data are presented as mean ± SEM from five to six samples for each condition unless noted. Significance is determined by ANOVA (P < 0.05).

### Results

#### Microscopic assessment unmodified and modified NPs

Scanning electron microscopy was used to assess the size and morphology of both unmodified and surface-modified NPs (Fig. 1). The average size for each batch of NPs was comparable (not statistically different) with mean particle diameters of 168 ± 28 nm (NP-Unmod), 137 ± 51 nm (NP-CH2.5), and 130 nm ± 56 nm (NP-CH20).

#### NP uptake using in vitro, ex vivo, and in vivo models

Using fluorescence microscopy, we visualized the internalization of NPs in bladder cancer cells after 2 hours (Fig. 2). Surface-modified NPs with chitosan (CH2.5 and CH20) had higher internalization than control NP-AV. We observed NPs outside the nucleus in the cytoplasm (Fig. 2A). FACS analysis verified that cells bind and internalize surface-modified NP-C6 after 2 hours. Avidin-modified NPs associated and internalized relative to untreated samples; however, the CH-modified NPs had superior internalization/binding. The internalization of modified C6 NPs was also quantified for T-24 and UM-UC-3 bladder cancer cells (Fig. 2B). The mean fluorescence intensity was the lowest for the cells treated with control NPs (modified only with avidin) and greatest for the cells treated with chitosan-modified NPs. Representative histograms illustrated a higher fluorescence intensity for UM-UC-3 cells treated with NP-C6-CH2.5 and NP-C6-CH20 compared with that of NP-C6-AV control (Fig. 2C).

During ex vivo experiments, NP formulations (2 mg/mL) were incubated with human ureter tissue for 30 to 120 minutes. At 60 minutes, the uptake of NP-C6-CH20,
NP-C6-CH2.5, and NP-C6-AP was 5, 4, and 3-fold greater than controls (NP-C6-Unmod or NP-C6-AV). Internalization of NP-C6-CH2.5 and NP-C6-CH20 after 120-minute incubation was 9 and 10-fold higher, respectively, than control NP-C6-AV (Fig. 3A).

An in vivo model showed that chitosan-modified NPs had the highest uptake after instillation into the mouse bladder. Relative to unmodified NPs, the amount of fluorescence doubled with NP-C6-AP and was 9 and 14 times greater with NP-C6-CH2.5 and NP-C6-CH20, respectively (n = 3–7; Fig. 3B).

**Dose–response curve and bioactivity of survivin**

UM-UC-3 bladder cancer cells were transfected with siSurvivin lipoplexes (Lipofectamine RNAiMAX), NP-siSUR-Unmod, NP-siSUR-AV, NP-siSUR-CH2.5, or...
NP-siSUR-CH20. We tested three different concentrations of siSurvivin (5, 50, and 100 nmol/L) to determine the most effective concentration needed to achieve survivin knockdown. Three days after administration of NP-siSUR (5 nmol/L), survivin mRNA expression decreased from 20% (NP-siSUR-Unmod; control) to 90% (NP-siSUR-CH20). Following administration of all surface-modified NPs containing siSUR at 50 and 100 nmol/L, survivin mRNA expression decreased by more than 95%. These results were comparable with the knockdown demonstrated using lipoplexes (Fig. 4A). To demonstrate prolonged knockdown, transfection of UM-UC-3 cells with 100 nmol/L siSurvivin, NP-siSUR-CH2.5, or NP-siSUR-CH20 resulted in 7-fold reduction in survivin mRNA expression relative to the controls, NP-siSUR-Unmod, or NP-siSUR-AV, after 9 days (Fig. 4B). Cell proliferation was measured in UM-UC-3 bladder cancer cells, which were treated for 2 hours with NP-siSUR-Unmod, NP-siSUR-AV, NP-siSUR-CH2.5, or NP-siSUR-CH20. Cell proliferation was reduced by 20% to 30% in the bladder cancer cells when treated with the chitosan-modified NPs compared with the NP-siSUR-Unmod control (Fig. 4C).
In vitro controlled release studies were performed with CH2.5- and CH20-modified NPs containing fluorescently labeled siSurvivin. Cumulative release of F-siSurvivin from the chitosan-modified NPs was measured in artificial urine over 13 days (Fig. 5). NP-siSUR-CH2.5 had greater initial burst release of F-siSurvivin within 24 hours, compared with NP-siSUR-CH20. The overall release of siRNA from CH2.5 NPs was 10 times greater than the release of siRNA from CH20 NPs.

**Tumor response to siSurvivin in a xenograft model**

Mouse tumors treated with NP-siSUR-CH2.5 were tumoristatic compared with those treated with PBS or empty NP-CH2.5 (Fig. 6A). Tumors treated with PBS or blank NPs increased 45% to 65% in size within 11 days following initial treatment. A gross view of excised bladder tumors with and without treatment is shown in Figure 6B. To quantify the level of survivin expression posttreatment, survivin mRNA from the xenograft mouse tumors was measured by qRT-PCR (Fig. 6C). Tumors treated with NP-siSUR-CH2.5 exhibited a 65% and 75% reduction in survivin expression compared with the PBS and NP-BkCH2.5, respectively.

**Discussion**

Treatments for interstitial cystitis/painful bladder syndrome, overactive bladder, and bladder cancer include electrical nerve stimulation, behavioral therapy, oral medications, transurethral resection, and/or intravesical instillations (1, 27, 28). Many current intravesical treatments for bladder disease involve repeated catheterizations due to short dwell time and poor adhesion to the bladder wall. Increased efficacy is likely with drugs or molecules that strongly adhere to the bladder wall (28). The repeated catheterizations may result in infection and irritation during voiding. Oral administration of oxybutynin for the treatment of overactive bladder and interstitial cystitis may be accompanied by side effects (29) that are mitigated when oxybutynin is administered intravesically (2, 30).

Polymeric NPs have the potential to enhance localized, intravesical delivery. PLGA NPs are attractive, nontoxic, biodegradable, and scalable vehicles for intracellular drug delivery. Furthermore, PLGA NPs encapsulate and provide controlled release of many drugs that would otherwise pose delivery issues to the bladder in an unencapsulated form. It has been reported that NPs can protect its cargo from degradation and enhance its stability (23). Also, by coencapsulating siRNA with spermidine, there is improvement in siRNA loading by 40-fold (9). In addition, PLGA NPs can be surface modified to provide enhanced cellular uptake, and better transport through the BPB. Therefore, PLGA NPs are excellent candidates for intravesical applications.

Because the BPB is a resilient barrier against most substances, we focused on two routes to transport NPs across the urothelium: transcellular (through the cells) and paracellular (through the tight junctions and lateral intercellular spaces). The objective of our study was to increase NP permeability through the urothelium of normal and diseased bladder tissue by using PLGA NPs surface modified with AP, a cell-penetrating peptide, and chitosan, a mucosalhesive polysaccharide. Recently, AP was shown to enhance internalization of PLGA NPs in epithelial cancer cells (31). In our human ureter model, the internalization of NP-AP was significantly greater than the NP-Unmod at 60 minutes. However, at 120...
minutes, NP-AP and NP-Unmod were not significantly different. We often see an increase in NP internalization up to a certain timeframe after which internalization decreases (32). This is dependent on a number of factors such as cell type (and cell uptake rate), number of receptors/ligands, process of NP uptake, surface modifications/characteristics of NPs. In our mouse bladder model, NP-AP was 2-fold more effective after 2 hours in penetrating the urothelium than NP-Unmod (Fig. 3B). In contrast with the moderate increase in internalization seen with NP-AP, urothelial cell internalization was greatly enhanced after 2 hours with NP-CH2.5 and NP-CH20. Nine- and 14-fold greater uptake was achieved with NP-CH2.5 and NP-CH20, respectively, relative to NP-Unmod. To our knowledge, this is the first study that uses low molecular weight CH-modified PLGA NPs to improve uptake using human ex vivo and mouse in vivo models.

In addition to enabling better cell internalization, surface-modified NPs also may enhance cell adhesion, which increases the likelihood of cell internalization in the fluid-filled bladder. We modified the surfaces of our NPs with chitosan to incorporate its favorable adhesion properties. Previously, chitosan had been shown to increase molecular adhesion in a variety of fluid-filled compartments (33). Bourlais and colleagues demonstrated that chitosan effectively increased NP residence time in the eye. Even in the presence of excess tear production, positively charged chitosan bound favorably to the negatively charged corneal surface of the eye (34). Fernandez-Urrusuno and colleagues showed that chitosan NPs increased nasal adsorption of insulin (35). In addition, chitosan is known to enhance tissue penetration by binding with molecules on the surface of the
urothelium, and disrupting the tight junctions between umbrella cells (36). In fact, by inducing desquamation of pig urothelium, chitosan removes all diffusion barriers, including glycosaminoglycans (GAG), membrane plaques, and umbrella cell tight junctions, thereby enabling deeper permeation. Because of its ability to increase residence time within fluid-filled compartments and enhance transport through urothelial tissues, chitosan is an attractive ligand for surface modification of NPs for intravesical instillation.

In designing these NPs, there was a balance between the adhesive properties of chitosan and the release of siRNA from the NPs, which are both affected by the molecular weight of chitosan (37). Eroglu and colleagues showed that higher molecular weight chitosan (650 kDa) had a greater bioadhesive force to sheep bladder than lower molecular weight chitosan (150 kDa; ref. 38). Although both chitosan NP formulations (CH2.5 and CH20) were effective in penetrating the urothelium of our human ex vivo and mouse in vivo systems, NPs with the longer CH20 chain showed stronger cellular uptake relative to NPs with the shorter CH2.5 chain. At acidic pH, chitosan, with abundant hydroxyl and amine groups, becomes protonated and forms electrostatic interactions with the GAGs found on the surface of the urothelium (1, 39, 40) or human cancer cells, thereby increasing chitosan’s binding potential. Moreover, Amoozgar and colleagues demonstrated that the longer chitosan chain had a five times greater positive surface charge than the shorter chitosan chain (40). The exact mechanism by which chitosan enhances penetration is unknown (1); however, enhanced internalization using chitosan-modified NPs is attributed to a combination of increased mucoadhesion and the temporary loss of the tight junctions (19, 36, 41, 42). Although the high molecular weight chitosan likely enhances binding and internalization, the positively charged chitosan can bind to the negatively charged siRNA preventing its release, so this must be considered in NP design.

The bioactivity of our NP-siSUR was measured by knockdown of survivin mRNA, an inhibitor of apoptosis. Previously, we showed that survivin is highly expressed in bladder cancer cells (22). In addition, we showed that survivin could be detected in urine of patients with bladder cancer, but was absent from the urine of patients without cancer (21). In fact, survivin expression is elevated in many cancers and absent in many noncancer tissues, making it a good target for cancer therapy (43). Yang and colleagues showed a 30% decrease in cell viability and proliferation of T-24 bladder cancer cells in vitro for 3 days when using survivin siRNA (44). We were interested in decreasing survivin expression by extending the bioactivity of siSurvivin. Previous studies showed that siSurvivin degradation typically occurs at 3 days when using commercially available transfection reagents (45). To test the efficacy of our NP-siSUR, we analyzed survivin expression in human bladder cancer cell models (Fig. 4). We initially varied the siSurvivin concentration to determine an efficacious dose. At higher concentrations of siRNA (100 nmol/L; ref. 46) in vitro, there was little difference between the chitosan formulations. After administration of 100 nmol/L siRNA, at day 9, we showed a 60% to 70% decrease in the expression of survivin using both chitosan formulations.

We then assessed the release of siRNA from NP-CH2.5 and NP-CH20 to determine the effect of surface modification on siRNA release. Although our in vitro data

![Figure 6.](https://www.aacrjournals.org/MolCancerTher;13(1)January2014OF9)
suggest that both NP-siSUR-CH2.5 and NP-siSUR-CH20 decrease survivin expression, likely due to enhanced internalization and subsequent release of siSurvivin, the overall release of survivin siRNA from NP-siSUR-CH2.5 was 10 times greater than that from NP-siSUR-CH20. This is in agreement with previous studies that show that the higher molecular weight (long chain) chitosan entangles and traps more free-siRNA than the lower molecular weight (short chain) chitosan (47), resulting in a hindrance of siRNA migration. Lower release of F-siSurvivin from NP-siSUR-CH20 was expected, relative to NP-siSUR-CH2.5, as the longer positively charged chains hinder siRNA release. Furthermore, longer chitosan chains can form bulkier surface layers and may affect siRNA release (38). Conversely, the short positively charged chitosan, NP-siSUR-CH2.5, demonstrated an initial burst release of the F-siSurvivin, followed by a steady release of siRNA. It has been reported that lower molecular weight chitosan has resulted in better transfection efficiencies, degradation, and intracellular release (48, 49). Because of this dramatically different release profile and transfection efficacy, NP-CH2.5 was used in the in vivo bladder tumor growth studies.

Using a xenograft mouse model, we demonstrated a significant reduction in tumor growth, up to 65%, with NP-siSUR-CH2.5, which was comparable with previous reports that used antisense oligonucleotide survivin or dominant-negative mutant survivin to reduce tumor growth by up to 50% (50). Kunze and colleagues delivered 4.6 mg/kg of free survivin siRNA 3 times per week in vivo, but did not observe a reduction in tumor growth (46), whereas we were able to deliver 20 times less siRNA in surface-modified NPs (only 2 times per week), with a significant reduction in tumor growth. We were able to show a greater decrease in tumor growth using our chitosan-functionalized NPs compared with other knockdown methods.

For the first time, we demonstrate that PLGA NPs, functionalized with low molecular weight chitosan encapsulating siRNA (and unmodified siRNA PLGA NPs) improves binding in human ex vivo specimens and in mouse in vivo specimens. Furthermore, the lower molecular weight CH2.5-modified PLGA NPs encapsulating siRNA prolonged survivin knockdown and reduced tumor growth. We demonstrated that chitosan-modified PLGA NPs have many advantages, including a sufficient amount of siRNA release to reduce survivin mRNA expression and an increase in siSurvivin bioactivity. We now have a versatile NP system that is capable of carrying large amounts of bioactive siRNA across the urothelium, thus increasing and improving therapeutic response.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.T. Martin, J.M. Steinbach, J. Liu, H.Z. Kaimakiotis, M.A. Wheeler, W.M. Saltzman, R.M. Weiss
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