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Characterization of a targeted nanoparticle functionalized with a urea-based inhibitor of prostate-specific membrane antigen (PSMA)

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Abstract

Polymeric nanoparticles represent a form of targeted therapy due to their ability to passively accumulate within the tumor interstitium via the enhanced permeability and retention (EPR) effect. We used a combined approach to decorate the surface of a nanoparticle with a urea-based small-molecule peptidomimetic inhibitor of prostate specific membrane antigen (PSMA). PSMA is expressed by normal and malignant prostate epithelial cells and by the neovasculature of almost all solid tumors. This strategy takes advantage of both the avidity of the functionalized nanoparticle for binding to PSMA and the ability of the nanoparticle to be retained for longer periods of time in the tumor due to enhanced leakage via EPR into the tumor interstitium. As an initial step to introducing the targeting moiety, the amino terminus of the small-molecule PSMA inhibitor was conjugated to PEG (M_n 3400) bearing an activated carboxyl group to obtain a PEGylated inhibitor. Studies undertaken using a radiolabeled PSMA-substrate based assay established that the PEGylated inhibitor had an IC_{50} value similar to the uncomplexed inhibitor. Subsequently, nanoparticles loaded with docetaxel were formulated using a mixture of poly(lactide- β -ethylene glycol- β -lactide) and PSMA-inhibitor bound α -amino- ω -hydroxy terminated poly (ethylene glycol- β - ϵ -caprolactone). In vitro studies using these nanoparticles demonstrated selective cytotoxicity against PSMA-producing cells. Binding of fluorescently labeled PSMA-targeted particles to PSMA-producing cells has also been directly observed using fluorescence microscopy and observed in secondary fashion using a PSMA substrate based enzyme inhibition assay. Ongoing in vivo studies address the localization, activity and toxicity of these targeted nanoparticles against PSMA-producing human prostate tumor xenografts.

Keywords

nanoparticle; docetaxel; polyethylene glycol; PSMA; prostate cancer; targeted

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in American men and remains uniformly fatal once it undergoes metastasis.¹ Androgen ablation therapy is effective palliative therapy, but in all men tumor progression eventually occurs even when completely androgen-deprived (e.g., inhibition of both testicular and adrenal androgens).² Traditionally, prostate cancer was thought to be relatively resistant to cytotoxic chemotherapies administered following androgen ablation.³ However, two recent studies demonstrated a modest survival benefit in men with hormone refractory metastatic disease treated with docetaxel.^{4,5} As with other cytotoxic therapies, docetaxel is associated with systemic toxicity that limits both the total dose and duration of therapy that can be administered.^{4,5} To improve the therapeutic window, a number of approaches have been explored to target cytotoxic agents like docetaxel selectively to tumor with the goal of higher tumor concentration and lessening of toxicity to normal tissues. In this regard, various prostate tissue specific surface proteins have been evaluated as potential binding targets to improve tumor uptake and retention of therapeutic agents.

The most extensively characterized surface protein has been prostate-specific membrane antigen (PSMA). PSMA is highly expressed by prostate cancer compared to most normal tissue.⁶⁻⁹ PSMA expression has also been demonstrated to increase following androgen ablation.^{10,11} Multiple studies have documented that PSMA is also expressed in the neovasculature of most solid tumors, but not in the vasculature of normal tissues.^{7,8} PSMA is a carboxy-peptidase and is relatively unique in its ability to function as both an *N*-acetylated alpha-linked dipeptidase and a gamma glutamyl (i.e., folate) hydrolase.^{12,13} Therefore, PSMA has been an attractive target for both targeted drug delivery and imaging. PSMA targeting approaches include the use of PSMA peptide substrates,¹⁴ PSMA-binding peptides,^{15,16} RNA aptamers^{17,18} or anti-PSMA monoclonal antibody-cytotoxin conjugates.¹⁹ Efforts have also been made to image PSMA-positive prostate tumors using labeled small-molecule peptidomimetic PSMA inhibitors^{20,21} and monoclonal antibodies.^{22,23}

Previously Zhou et al. reviewed a series of urea-based PSMA inhibitors with high picomolar to low nanomolar K_i values.²¹ Radiolabeled versions of these inhibitors have been used to selectively image PSMA-expressing prostate cancer xenografts.²⁰ On the basis of these studies, we developed an approach to functionalize nanoparticles with a highly potent urea-based PSMA inhibitor which could enable homing of the nanoparticle to prostate cancer. The small-molecule inhibitor would allow for the generation of a highly decorated nanoparticle surface in which multiple ligand-protein binding interactions would produce an avidity effect that would enhance the binding of the nanoparticle to PSMA.

In previous studies, Farokhzad et al demonstrated that docetaxel encapsulated into poly(lactide- β -ethylene glycol- β -lactide) (PLA-PEG-PLA) nanoparticles and conjugated to PSMA binding aptamer produced an antitumor effect when injected intratumorally into PSMA expressing prostate cancer cells.¹⁷ PLA-PEG-PLA is often chosen as the controlled release system because its component polymers have been previously demonstrated to be biocompatible and have been extensively used in drug development.²⁴⁻²⁸ As a novel modification of this commonly used strategy, we introduced a functionalized PEG arm as a spacer using an α -amino- ω -hydroxy terminated poly(ethylene glycol-*b*- ϵ -caprolactone) (PEG-PCL) polymer chain in order to maintain sufficient distance between the small-molecule PSMA inhibitor and the nanoparticle surface. PEG functions in this targeting application to decrease nonspecific protein binding (i.e., 'bio-fouling') and minimizes particle clearance by the reticuloendothelial system.²⁵ Additionally, the PEG-PCL should enhance partitioning and presentation of the targeting ligand on the surface of the

nanoparticle because the orientation of PEG + hydrophilic ligand towards the surface of the nanoparticle is energetically highly favorable.

Using this rationale, a strategy was developed to generate a PEGylated urea-based PSMA inhibitor incorporated into a PLA-PEG-PLA nanoparticle, as shown in Figure 1. The components of this system and the PSMA inhibitor conjugated nanoparticles were then characterized for their ability to inhibit the enzymatic activity of PSMA. Subsequently, docetaxel encapsulated, PSMA-targeted nanoparticles were evaluated for their ability to bind to PSMA expressing human LNCaP prostate cancer cells and to selectively inhibit their growth in vitro.

Results

Binding of PEGylated inhibitor, FPPi, to PSMA

The PSMA inhibitor PSMAi1 is ideally suited for this nanoparticle application due to the presence of a primary amine that allows for amide bond formation with carboxyl groups present on PEG monomers. Previously it had been demonstrated that urea-based PSMA inhibitors bearing structural homology to PSMAi1 inhibited NAAG hydrolysis with the IC₅₀ value range of 1–10 nM.²⁹ On this basis, PSMAi1 was coupled to fluorescently labeled 3400 MW PEG as outlined in Figure 6, Scheme 1. The presence of the fluorescein at the opposite end of the PEG monomer allowed us to follow the reaction and subsequent purification of the product by dialysis. FPPi inhibited PSMA hydrolysis of NAAG with an IC₅₀ value between 1 and 10 nM (Fig. 2A). While we expected the addition of the large PEG moiety to increase the IC₅₀ value, the IC₅₀ value was still in the same range suggesting that (a) the addition of PEG did not pose a steric hinderance and (b) FPPi possessed sufficient affinity for PSMA to justify further studies in which the PEGylated inhibitor would be introduced onto the surface of nanoparticles. PEG itself was not observed to bind to affect the enzymatic of PSMA (data not shown).

Nanoparticle formulation

The synthesis of the PSMA-targeted nanoparticle was based on a strategy whereby the PLA-PEG-PLA copolymer partitions such that the PLA region would comprise the core of the nanoparticle while the water-soluble but acetone-insoluble PEG region would be in the aqueous layer and thus emerge on the surface. This approach has been defined earlier and provides stealth attributes to the nanoparticle.^{17,30} Since the tricarboxylic acid containing PSMA inhibitor is highly hydrophilic, it would be expected to orient itself toward the surface of the nanoparticle during formulation. In order to facilitate such orientation, it was conjugated to the PEG-PCL copolymer such that the hydrophobic PCL would integrate into the nanoparticle matrix and the PEG and hydrophilic PSMAi2 would orient toward the surface. On this basis, the polymer bound PSMA inhibitor (polyPSMAi2) was synthesized as outlined in Figure 6, Scheme 2A. ¹H-NMR and ESI analysis confirmed correct structure. Nanoparticles incorporating polyPSMAi2 (nanoPSMAi2) and docetaxel were then formulated using the solvent evaporation technique. NanoPSMAi2 particles were found to be monodisperse with a polydispersity index of 0.131 and had an average size of approximately 222 nm, Figure 3. The loading efficiency of docetaxel was observed to be in the range of 40 ± 2% over multiple experiments. The surface density of the PSMA inhibitor was computed to be 2.23×10^{17} molecules/m² of surface area of the nanoparticles (~30,000 inhibitor molecules/nanoparticle).

Nanoparticle binding as observed by NAAG assay

Nanoparticles formulated with a surface decorated with small-molecule PSMA inhibitors were tested for activity against PSMA from LNCaP homogenate via the NAAG assay as

described above. Initial testing was carried out with polyPSMAi2 which was observed to inhibit the activity of PSMA at an IC_{50} of close to 1000 ng/mL. Subsequently, polyPSMAi2 was incorporated into the nanoparticle matrix to give nanoPSMAi2, which was also observed to inhibit PSMA activity. A shift of the binding curve to the right was observed, Figure 2B, suggesting that when tethered on the surface of the nanoparticle, the inhibitor is unable to bind to PSMA with the same affinity as it could when in the polymeric form.

Observation of nanoparticle binding by fluorescence

To evaluate whether the nanoPSMAi2 particles exhibited enhanced binding to PSMA-positive prostate cancer cells compared to nontargeted nanoPEG particles, we labeled the respective particles with Texas Red. This was accomplished by incorporating a Texas Red labeled PEG-PCL copolymer (polyTR) into the nanoparticles. The incorporation of Texas Red (MW 625 Da) labeled polymers was not expected to alter the size of the nanoparticles significantly. Since it is known that cells can nonspecifically endocytose polymer nanoparticles,³⁴ the concentration of the nanoparticles was kept low. The cells were also incubated with the nanoparticle suspension under agitation to further minimize endocytosis.

A confocal section of the nontargeted Texas Red labeled nanoPEG particles exhibited no red fluorescence as would have been observed with nanoparticles binding to cells, Figure 4A. In contrast, the targeted nanoPSMAi2 particles undergo endocytosis after 15 min of incubation, Figure 4B. Previously it was demonstrated that PSMA becomes internalized following binding by antibodies and small-molecule PSMA inhibitors.³⁵ PSMA can also undergo internalization and be recycled in the absence of ligand binding. Herein, minimal to no endocytosis was observed in the case of the nontargeted particles. Thus, it appears that the endocytosis is mediated by nanoPSMAi2 particle binding to PSMA leading to subsequent internalization by endocytosis.

In addition to confocal microscopy, fluorescence microscopy was undertaken with the intention of observing nanoparticle binding on the cell surface. Figure 4C and D suggest that binding on the cell surface is highly dependent on the presence of a targeting moiety on the surface of the nanoparticle. As expected, minimal nonspecific surface binding is observed in the case of the nontargeted nanoparticles. In contrast, a high number of PSMA-targeted nanoparticles are seen on the cell surface away from the nuclei.

In vitro cytotoxicity to PSMA expressing human prostate cancer cells

To evaluate whether incorporation of the urea-based PSMA inhibitor enhanced the cytotoxicity of the nanoparticle to PSMA expressing cells in vitro, we compared the effects of the docetaxel loaded nanoPSMAi2 particles to those of the untargeted nanoPEG particles and docetaxel alone. Following determination of the amount of docetaxel loading in the particles, PSMA-expressing LNCaP human prostate cancer cells were exposed to equimolar amounts of loaded nanoparticles or free docetaxel at concentrations of 10 and 100 nM. To minimize the effects of nonspecific endocytosis, cells were only exposed to test compounds for 15 min at which time cells were washed and then placed in drug free media. Cell counts were determined by converting absorbance from MTT assay to cell number based on a standard curve of absorbance from MTT assay of known amounts of LNCaP cells. In this assay, 48 hrs following exposure, no antitumor efficacy was observed at a concentration of docetaxel of 10 nM. In contrast, the growth of cells exposed to 100 nM docetaxel equivalents was inhibited by 65% for the nontargeted nanoPEG particles while cell growth was inhibited 89% by docetaxel and 95% by the nanoPSMAi2 particles. At 96 hrs after exposure to the nontargeted particles cells continued to grow but overall cell growth was inhibited 70% compared to control. In contrast, at this time point exposure to the nanoPSMAi2 particles resulted in a ~10% decrease in the absolute cell number compared to

starting cell number at day 0, Figure 5. These results demonstrate the increased antitumor efficacy that can be achieved by incorporating a cell surface protein specific binding ligand onto the surface of a docetaxel encapsulated nanoparticle.

Discussion

In these studies we have established that a nanoparticle system decorated with a small-molecule PSMA inhibitor on the surface enhances binding to PSMA. Our approach is comparable to the approach of Farokhzad et al. who characterized a docetaxel encapsulated PSMA-targeted RNA aptamer based nanoparticle for in vitro toxicity and for efficacy following intratumoral injection in vivo.¹⁷ Those authors selected a previously described RNA aptamer A10, which is a competitive inhibitor with a reported K_i for PSMA of 11.9 nM.¹⁸ The urea-based inhibitor used to target our particles has a K_i value in a similar range. In this earlier study, Farokhzad et al. compared the in vitro cytotoxicity of docetaxel encapsulated PSMA-targeted RNA aptamer nanoparticle to a nontargeted nanoparticle to LNCaP cells.¹⁷ This group demonstrated that 72 hrs after a 30 min exposure the viability of LNCaP cells exposed to the PSMA-targeted particles was 20% lower than similar particles lacking the targeting aptamer.¹⁷ The cytotoxicity of the nontargeted particles in that study was ascribed to a combination of nonspecific uptake and to the expected nonspecific release of docetaxel from the particle into the media over the exposure period. In our study, using a similar MTT-based assay, we demonstrate that 96 hours following a 15 minute exposure to the PSMA-targeted docetaxel loaded nanoparticles, the total cell number compared to day zero decreases by ~10%. In contrast, 96 hours after exposure to the non-targeted docetaxel nanoparticles the total cell number increases by ~250% compared to day zero.

Using a small-molecule inhibitor based system has certain unique advantages. First, the chemistry can be more controlled because unlike antibodies or aptamers, small-molecules can be synthesized with higher purity, accuracy, efficiency and economy. Another reason as compared to antibodies and aptamers is that unlike antibodies, small-molecules do not need to form tertiary structures to facilitate binding. Thus stability during formulation, which can be a big factor, is not an issue in this case. The use of a small-molecule inhibitor is advantageous since it has a lower exclusion space suggesting that up to 30,000 inhibitor molecules can be loaded on the surface (as observed in this study) thus maximizing the possibility of binding. And finally, in terms of future in vivo experiments, small-molecules are less likely to elicit an immune response compared to large antibodies, and therefore such nanoparticles should provide long circulatory half lives in the body upon injection.

In summary, the results demonstrate that PSMA-targeted nanoparticles enhance cytotoxicity via binding to PSMA protein present on the surface of prostate cancer cells. On this basis, further in vivo studies are warranted to assess the effectiveness of the PSMA-targeted nanoparticles.

Materials and Methods

Fluor-PEG-NHS (Nektar Therapeutics, AL) was used as purchased. All other polymers were purchased from Polymer Source (Canada). They were used as instructed by the manufacturer. Tetronic 904 surfactant was a kind gift from BASF (Florham Park, NJ). Unless otherwise described, all chemicals were purchased from Sigma Aldrich (Saint Louis, MO), and used without any further purification.

Cell lines

LNCaP human prostate cancer cell lines used in this study were purchased from ATCC and grown in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, UT), 1%

penicillin-streptomycin (Mediatech, VA), and 1% L-glutamine (Mediatech, VA). The cells were maintained at 37°C in a humidified 5% CO₂-containing incubator.

Synthesis of PEGylated PSMA inhibitor (FPPi)

2-[3-(5-amino-1-carboxypentyl)-ureido]-pentanedioic acid (PSMAi1) was synthesized according to previously described methods.²⁹ To a solution of this compound (38 mg, 88.2 µmol in 1.5 mL dimethylformamide) was added diisopropylethylamine (0.3 mL, 1.72 mmol) followed by Fluor-PEG-NHS (MW ~3400) solution (150 mg, 44.11 µmol in 1 mL dimethylformamide) at 0°C, Figure 6, Scheme 1. After 5 min, the solution was allowed to warm to room temperature and was kept for 16 h under an argon atmosphere. The solution was concentrated under high vacuum to yield a yellow residue. The residue was dissolved in 10 mL of methylene chloride and washed with 5 × 10 mL of water to remove the starting material. Organic fractions were combined and concentrated under reduced pressure to yield a yellow solid. The solid FPPi was further purified by dialysis against water using a MW 1000 cutoff membrane (SpectraPOR CE, CA) using 3 solvent changes over 4 days, followed by lyophilization for further use. FPPi was characterized by ¹H-NMR.

Synthesis of NHS ester of PSMA inhibitor, 2-(3-{1-carboxy-5-[7-(2,5-dioxo-pyrrolidin-1-ylloxycarbonyl)-heptanoylamino]-pentyl}-ureido)-pentanedioic acid (PSMAi2)

2-{3-[5-[7-(2,5-dioxo-pyrrolidin-1-ylloxycarbonyl)-heptanoylamino]-1-(4-methoxybenzyloxycarbonyl)-pentyl]-ureido}-pentanedioic acid bis-(4-methoxy-benzyl) ester (30 mg, 0.032 mmol) was synthesized according to previously described methods.²⁹ This compound was dissolved in 5 mL 1:1 TFA:methylene chloride solution and was kept at room temperature for 3 h, Figure 6, Scheme 2A. The resulting solution was evaporated under reduced pressure. The colorless solid residue was washed 5 × 1 mL of diethyl ether. The residue was dissolved in 10 mL chloroform and extracted with 3 × 10 mL water to remove impurities. The organic layer was evaporated to dryness to get the desired product, PSMAi2. Yield: 11.4 mg, 62%. Product was characterized by ¹H NMR and MALDI-TOF.

Coupling of PSMAi2 to PEG-PCL (polyPSMAi2)

PSMAi2 (11.4 mg, 20 µmol) was dissolved in 3 mL dimethylformamide (DMF). PEG-PCL (MW~25000, ratio of PEG:PCL is approximately 1:4 by weight)(257 mg, 10 µmol in 5 mL DMF) was added at room temperature followed by diisopropylethylamine (0.5 mL) and was kept at room temperature for 48 hr, Figure 6, Scheme 2B. The resulting solution was concentrated under vacuum and a white solid was then precipitated by dropwise addition to water. The precipitate was redissolved in DMF, precipitated again in water and then washed with 5 × 10 mL methanol. All organic fractions were combined together and evaporated under reduced pressure. The desired product was obtained as a colorless solid and characterized by ¹H-NMR.

Synthesis of texas red labeled PEG-PCL (polyTR)

115 mg (4.6 µmol) of PEG-PCL was dried overnight under high vacuum. Texas Red sulfonyl chloride (3 mg, 4.8 µmol in 1 mL of dry DMF and 0.1 mL diisopropylethylamine) was then added to PEG-PCL under N₂ with vigorous stirring. The reaction was allowed to proceed overnight in the dark at room temperature. Separation of the labeled copolymer was achieved by precipitation in water. Further purification was achieved by redissolving in DMF followed by reprecipitation and washing with 5 × 10 mL of water. Final product, polyTR was obtained as a lyophilized solid and characterized by fluorescence.

Formulation and characterization of nanoparticles

Polymer nanoparticles were formulated as a modification of the solvent evaporation technique as described previously.³⁰ Briefly, nanoparticles were prepared by initially dissolving 5 μ L of 100 mg/mL docetaxel in DMSO into 5 mL of a 10 mg/mL solution of PLA-PEG-PLA. PLA-PEG-PLA comprised of a 1.2 kDa chain of PEG flanked by a 6.3 kDa chain of PLA on either side. The polymer was nanoprecipitated along with the encapsulated drug by slowly pipetting into 10 mL of a 0.4% solution of Tetronic 904 with vigorous agitation for approximately 15 min. Subsequently, the acetone was evaporated under a rotary evaporator taking care to prevent excessive bumping in the round bottomed flask. The polymer nanoparticles were separated by centrifuging at 10000 g for 15 min. This was repeated once. The nanoparticles were finally redispersed in Tetronic 904 depending on the desired final concentration of docetaxel. Targeted nanoparticles were formulated in a similar fashion with the exception that the polyPSMAi was incorporated into the nanoparticle by initially dissolving it with PLA-PEG-PLA in acetone such that a known ratio of polyPSMAi to PLA-PEG-PLA was maintained. When fluorescent particles were desired, polyTR was added in a manner akin to the polyPSMAi.

Particle size was determined using a Zetasizer (Malvern Zetasizer 3000, Malvern, UK). Each analysis lasted until a suitable value for the auto-correlation function was obtained and was performed at 25°C. The measurement was repeated three times with the average size across the measurements being reported.

Surface coverage of PSMA inhibitor on the nanoparticles was determined by ¹H-NMR. Targeted nanoparticles were dissolved in CHCl₃ and NMR spectra were obtained. The ratio of the proton peak at 5.2 ppm from the polycaprolactone to the peak at 4.1 from the polylactic acid provided us with a measure of integration of the polyPSMAi into the PLA-PEG-PLA copolymer mesh. Given that the yield of the reaction in which the PSMA inhibitor was conjugated to PEG-PCL was previously evaluated, it was possible to determine the number of PSMA inhibitor molecules per gram of polymer. With the assumption that the particles have a specific gravity of one, and with knowledge of the particle size, the surface coverage can be computed.

Determination of drug loading

High performance liquid chromatography (HPLC) analysis was carried out using a C-18 column on a reversed phase HPLC system (Waters, MA). A dual-absorbance detector (Waters 2457) was used for analysis at 215 nm and 254 nm. The mobile phase consisted of solvent A, 0.1% TFA in water and solvent B, 0.1% TFA in acetonitrile. Gradient elution from 20% B to 100% B over 22 minutes was used for chromatography. Chrom Perfect software supplied with the HPLC system was used for data analysis. A calibration curve for docetaxel was initially generated by injecting known amounts of docetaxel and measuring the area under the elution curve at 254 nm. The area under the curve was fitted to the injection amount in a linear fashion. To determine the amount of encapsulated drug in the nanoparticles, a known amount of drug-loaded nanoparticles was dissolved in DMSO followed by injection onto the HPLC. The area under the curve of the elution profile corresponding to docetaxel was measured and the amount of docetaxel was estimated using the standard curve. Using the initial amount loaded onto the nanoparticles, the loading efficiency was computed.

N-acetylated-aspartyl-[³H]glutamic acid (NAAG) assay for PSMA activity

The NAAG assay involves measuring the cleavage of 50 nM NAAG by PSMA as defined in earlier work,³¹ in which the tritiated glutamic acid is released after hydrolysis. The reaction was carried out in a microfuge tube. At the desired time point, a known volume of the

NAAG containing released [³H]glutamic acid was added to an ion-exchange column. The released [³H]glutamic acid binds to the column and is eluted by using 0.1 M formic acid. Degree of release of [³H]glutamic acid was measured by scintillation counting and is a direct measure of the release of product after enzymatic reaction. LNCaP cell homogenate containing 2.8 ug/mL of protein was used as the source of PSMA for inhibition studies. PSMA-containing LNCaP homogenates were incubated with either inhibitor, nontargeted nanoparticles or targeted nanoparticles. The IC₅₀ value, as obtained from this study, is a measure of 50% inhibition of the activity of PSMA as compared to the activity in the absence of inhibitor.

Confocal microscopy

LNCaP cells were incubated with Cell Tracker Green (Molecular Probes, OR) at a concentration of 5 mM in buffer for 15 min, after which they were washed with media to remove unreacted dye. The cells were then stripped off the flask by using a final concentration of 2.5 mM of EDTA for 15 min. Cells were collected, spun down, resuspended in media and stored in the incubator for 15 min followed by incubation with targeted or untargeted nanoparticles. Both nanoparticles were labeled with Texas Red. The degree of fluorescence from both nanoparticle systems was estimated by measurement of fluorescence of a known concentration of nanoparticles under a fluorescence plate reader (DTX880, Beckman Coulter, CA). Cells were separated from the nanoparticles by centrifuging at 300 g for 5 min. Spinning was repeated to remove all unbound nanoparticles from the cell suspension, following which cells were plated on glass bottom petridishes (MatTek Corporation, MA) and visualized using a Utraview LCI (Perkin Elmer, MA) confocal microscope equipped with Spinning Nipkow disk with microlenses. Cells were viewed using a 100X objective. Images were captured in the temporal module using a LSI-cooled 12-bit CCD camera at 488 nm and 654 nm respectively. Images were processed using the NIH ImageJ software (<http://rsb.info.nih.gov/ij/download.html>).

Fluorescence microscopy

LNCaP cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, CA) for 5 min following which the excess dye was removed. Further incubation with nanoparticles was undertaken as described above for confocal microscopy. Cells were mounted on slides followed by imaging using a Nikon Eclipse E800 system (Santa Clara, CA) under 100X magnification. Three representative fields per slide with two slides for each condition were used.

In vitro toxicity of nanoparticles

Cytotoxicity assays were performed as described previously.³² Approximately 20,000 LNCaP cells were seeded in 96-well plates and permitted to attach for two days. These cells were then incubated with 100 µL of a suspension of docetaxel loaded nanoparticles to a final concentration of 100 nM docetaxel. After a 15 min exposure, the media and nanoparticles in all of the wells were carefully removed by pipetting to minimize cell detachment, followed by gentle washing with 200 µL of media. Cells were then incubated in media at 37°C and at the end of 48 hours and 96 hours, effects on cell growth were determined using the MTT assay (Promega, WI) according to manufacturer's instructions.

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Figure 1.

A general schematic demonstrating the binding of the nanoparticle. PSMA in dimeric form is observed on the cell surface. The polymeric nanoparticle has multiple PEG arms, some of which have the PSMA inhibitor attached to it. Total surface coverage by the inhibitor was computed to be 2.23×10^{17} molecules/m² of surface area of the nanoparticles (~30,000 inhibitor molecules/nanoparticle).

Figure 2.

(A) Inhibition of activity of PSMA by FPPi and PEG. FPPi inhibits the activity of PSMA with an IC₅₀ in the range of 10 to 100 ng/mL. In contrast, PEG inhibits the activity of PSMA by no more than 10% at its highest concentration of FPPi tested suggesting specific inhibition (data not shown). (B) PSMA inhibition by polyPSMAi2 when in free and nanoparticle form. There is a slight increase in the IC₅₀ when bound to the nanoparticle due to steric hindrance.

Figure 3. Size distribution of nanoPSMAi2 as obtained by Light Scattering. The number average of size was estimated to be 222 nm. As observed, the nanoparticles are monodisperse with a polydispersity index of 0.131.

Figure 4.

(A) Confocal microscopy using cells stained with Cell Tracker Green shows minimal endocytosis of nontargeted nanoparticles (red). (B) In contrast, PSMA-targeted nanoparticles have been endocytosed (red). (C) Fluorescence microscopy using DAPI nuclear stain also shows minimal binding of red nontargeted nanoparticles. (D) However, in case of targeted nanoparticles, multiple particles (red) are seen bound to the cell surface. Images are representative of the entire field.

Figure 5.

In vitro toxicity of nontargeted and targeted nanoparticles after a 15 min incubation at a docetaxel concentration equivalent of 100 nM. After 48 hour incubation, the treated arms show regression in growth. The effect is magnified with the higher incubation time of 96 hours where the controls have expanded considerably, the nontargeted nanoparticles demonstrate regression and the PSMA-targeted nanoparticles not only induce regression, but also reduce the overall cell number. Docetaxel was used as the control.

Figure 6.

Scheme 1. Synthesis of the PEGylated PSMA inhibitor, FPPi. Scheme 2. Synthesis of the PSMA inhibitor, PSMAi2 followed by synthesis of the polymeric version of the PSMA inhibitor, polyPSMAi2.