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Prostate cancer-specific thermo-responsive polymer-coated iron oxide nanoparticles

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ABSTRACT

Thermo-responsive poly(*N*-isopropylacrylamide-acrylamide-allylamine)-coated magnetic nanoparticles (PMNPs) were developed and conjugated with prostate cancer-specific R11 peptides for active targeting and imaging of prostate cancer. The stable nanoparticles with an average diameter of 100 nm and surface charge of −27.0 mV, had a lower critical solution temperature of 40 °C. Magnetic characterization showed that the nanoparticles can be recruited using a magnetic field and possess superparamagnetic behavior even after R11 conjugation. *In vitro* cell studies demonstrated that R11-conjugated PMNPs (R11-PMNPs) were compatible with human dermal fibroblasts and normal prostate epithelial cells to all tested concentrations up to 500 μg/ml after 24 h of incubation. Moreover, the nanoparticles were taken up by prostate cancer cells (PC3 and LNCaP) in a dose-dependent manner, which was higher in case of R11-PMNPs than PMNPs. Further, *in vivo* biodistribution of the nanoparticles showed significantly more R11-PMNPs accumulation in tumors than other vital organs unlike PMNPs without R11 conjugation. Moreover, R11-PMNPs decreased 30% magnetic resonance T2 signal intensity in tumors *in vivo* compared to 0% decrease with PMNPs. These results indicate great potential of R11-PMPs as platform technology to target and monitor prostate cancers for diagnostic and therapeutic applications.

1. Introduction

Prostate cancer is a commonly diagnosed cancer and the second leading cause of cancer-related deaths in US men. Common treatments for prostate cancer such as surgery, hormone therapy, radiation therapy, and chemotherapy are still not able to cure this disease and are also associated with different adverse side effects [1]. The complications associated with surgery are pain, urinary incontinence, and the possibility of permanent impotence. The side effects of hormone therapy include loss of sexual desire, impotence, and hot flashes leading to poor quality of life for prostate cancer patients. Radiation therapy causes tiredness, diarrhea, uncomfortable urination, and hair loss in the pelvic area. For chemotherapy, systemic toxicities are hair loss, weakness, immuno-suppression, and weight loss [1,2]. In general, limitations of conventional treatments are associated with non-specific targeting of therapeutic modalities. Alternative therapeutic modalities such as targeted therapy with cancer specificity would be a better treatment to enhance therapeutic efficacy in prostate cancer patients.

Compared to passive targeting, active targeting mechanisms such as receptor-mediated targeting or magnetic targeting further enhance the efficacy of drug delivery vehicles. Receptor-mediated targeting has extensively utilized targeting ligands such as RGD and folic acid to target the prostate cancer [3,4]. RGD is specific for $\alpha_v\beta_3$ integrin molecules in tumor angiogenesis, whereas folic acid is specific to folate receptors over-expressed on tumor cells. However, these targeting ligands can find their targets in other cancer types as well. One strategy to increase the specificity of biological targets

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is to use a cancer-specific ligand, including cell-penetrating peptides (CPPs), to introduce a drug delivery vehicle into the cell [5]. CPPs are capable of crossing cell membrane via macropinocytosis mechanism that delivers CPPs to the intracellular systems [6]. Of the commonly used CPPs, arginine-rich CPPs including HIV-Tat peptides and oligoarginines have been reported with high internalization efficacy [7]. We have unveiled a polyarginine peptide (R11) that has a prostate cancer specificity, which can be used as a prostate cancer imaging probe [8–10].

To overcome limitations of systemic chemotherapy, several carriers such as liposomes, dendrimers, and polymeric nanoparticles have been developed to encapsulate anticancer drugs and deliver them to the tumors. The US Food and Drug Administration (FDA) approved drug delivery nanoparticles and liposomal formulations are PEGylated liposomal doxorubicin (Doxil), liposomal daunorubicin (DaunoXome) and albumin bound paclitaxel nanoparticles (Abraxane) [1]. Yet one of the major limitations of these carriers is that it is not possible to monitor the distribution of drugs and the progress of treatment in real time. Since knowledge of the biodistribution of drug formulations is a key to their successful development for tumor targeting, drug carriers that can also be used as tracers or contrast reagents are needed for the development of effective alternative cancer therapies. Magnetic-based theranostic nanoparticles that simultaneously deliver both imaging and therapeutic agents are of great interest in cancer management. Previously, we have developed thermo-responsive poly(*N*-isopropylacrylamide-acrylamide-allylamine) (PNIPAAm-AAm-AH)-coated superparamagnetic iron oxide magnetic nanoparticles (MNPs) for controlled drug delivery applications [11,12]. We have shown that PNIPAAm-AAm-AH-coated MNPs (PMNPs) release therapeutically effective drugs in response to the changes in temperature. PMNPs exhibit lower critical solution temperature (LCST) at ~40 °C and carry amine functional groups for bioconjugation. In this study, R11 peptides were conjugated to PMNPs for actively targeted drug delivery for prostate cancer therapy. We hypothesize that R11-conjugated PMNPs (R11-PMNPs) will efficiently target prostate cancer and monitor tumor response to treatment using non-invasive imaging modalities.

2. Experimental section

2.1. Materials

All the chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification, if not specified. Iron oxide MNPs (Meliorum Technologies, Rochester, NY), acetic acid (EM Science, Gibbstown, NJ), hydrochloric acid (HCl, EMD Chemicals Inc., Gibbstown, NJ), ethanol (Fisher Sci., Fair Lawn, NJ), and epoxy gel (Loctite Corp., Rocky Hill, CT) were purchased and used as received. All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell culture media, media supplements, and Picogreen DNA assay were purchased from Invitrogen Corp. (Carlsbad, CA).

2.2. Synthesis of PMNPs and R11-PMNPs

MNPs were first coated with vinyltrimethoxysilane, a silane-coupling agent, by acid catalyst hydrolysis and followed by electrophilic substitution of ferrous oxide on the surface of MNPs as reported previously [11]. The silane–MNPs (28 mg) were then used as a template to polymerize NIPAAm (100 mg), AAm (13 mg) and AH (26.25 mg) while sonicating at 50 W for 30 min in the presence of deionized (DI) water *N*,*N*′-methylenebisacrylamide (13 mg), sodium dodecyl sulfate (41 mg), ammonium persulfate (APS, 78 mg), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (78.28 mg). The reaction was carried out under nitrogen for 4 h at room temperature. The PMNPs were collected by a magnet, and washed several times with DI water to remove surfactants and unreacted chemicals. R11 peptides (10 μM) were further conjugated to PMNPs via carbodiimide chemistry for receptor-mediated targeting of prostate cancer, as described previously [13]. The R11-PMNPs were then collected using a magnet and the supernatant was collected to calculate conjugation efficiency of R11 to the PMNPs using UV–Vis spectrometer (Tecan Ltd., Durham, NC).

2.3. Characterization of nanoparticles

Nanoparticles were characterized for their size and structure using a transmission electron microscope (TEM; Technai, JEOL 1200 EX, Tokyo, Japan). Hydrodynamic diameter, polydispersity index (PDI), and surface charge on the nanoparticles were characterized using zeta potential analyzer (ZetaPALS, Brookhaven Instruments, NY) with dynamic light scattering (DLS) detector. The LCST of the polymer and magnetic recruitment of the PMNPs were characterized and recorded by taking pictures as described previously [11,14]. Further, the iron content in the nanoparticles was evaluated using an iron assay as described elsewhere [4]. In brief, nanoparticles (1 mg/ml) were incubated with 30% v/v HCl at 55 °C for 2 h. APS (50 μg) was then added and after 15 min of shaking, potassium thiocyanate (50 μl, 0.1 M) was added, followed by shaking for another 15 min. The sample absorbance was read at 520 nm using UV–Vis spectrometer and compared against standard concentrations of iron oxide nanoparticles. Moreover, the magnetic properties of the nanoparticles were studied using a vibrating sample magnetometer (VSM; EV7, KLA-Tencor, Milpitas, CA) and hysteresis loops for PMNPs and R11-PMNPs were obtained at room temperature and compared with that of bare MNPs.

2.4. In vitro cell studies

The cytotoxic effects of free R11 peptides and R11-PMNPs were studied on human dermal fibroblasts (HDFs) and normal prostate epithelial cells (PZ-HPV-7). The cells were seeded at a density of 5000 cells/well in 96-well plates. After 24 h of seeding, the culture medium was replaced with medium containing either R11 peptides (0.1, 1, 5 and 10 μM) or R11-PMNPs (0, 50, 100, 250 and 500 μg/ml). The cells were incubated for 6 and 24 h, followed by the addition of MTS reagent, and the cell viability was determined following the manufacturer's instructions (Promega, Madison, WI). Further, to study the cellular uptake of nanoparticles, prostate cancer cells (PC3 and LNCaP) were seeded in 48-well plates and incubated for 24 h. The cell medium was then replaced with medium containing nanoparticles at various concentrations (0, 50, 100, 200, 300 and 500 μg/ml). After 2 h of incubation, the medium was removed and cells were washed several times with phosphate buffer solution (PBS), followed by lysis using 1% Triton X-100 (MP Biomedicals Inc., Solon, OH) in PBS. The iron contents internalized by cells were analyzed by iron assays and normalized with the total DNA content, assessed using Picogreen DNA assays as per manufacturer's instructions.

2.5. In vivo biodistribution of nanoparticles

Animal studies were performed in compliance with guidelines set by the University of Texas Southwestern Institutional Animal Care and Use Committees. Male NOD SCID mice (6–8 weeks of age) were purchased from the University of Texas Southwestern mouse-breeding core (Wakeland Colony). Preliminary *in vivo* biodistribution studies were conducted to evaluate the tumor specificity of R11-PMNPs in comparison with PMNPs. First, to test the time required for maximum accumulation of nanoparticles in prostate, PMNPs (100 μl, 5 mg Fe/kg) were injected intravenously via tail veins of mice. After 1, 4 and 24 h post injection, the animals were sacrificed and the vital organs (kidney, liver, spleen, lung and prostate) were excised. The tissue sections were stained using Prussian blue staining to detect the presence of iron [15].

Further, prostate cancer xenograft models were created by injecting PC3-KD cell suspension subcutaneously (5×10^5 cells/site, injection volume 100 μl) into both flanks of the animals as described elsewhere [9]. The animals were monitored three times a week and further studies were performed when the tumors became palpable. To determine the biodistribution and tissue-specificity of nanoparticles, saline or nanoparticles (100 μl, 5 mg Fe/kg) were injected intravenously (i.v.) via tail veins of the animals. After 24 h (from previous time study) of injection, the animals were sacrificed and the vital organs (kidney, liver, spleen, lung, prostate and tumor) were excised. The tissue sections were stained using Prussian blue staining. The area covered by the Prussian blue stain was analyzed quantitatively using ImageJ software (NIH, Bethesda, MD). In addition, iron assay was performed on the excised organs to quantify the amount of iron present in each organ. Briefly, a fixed amount of each organ was mixed with lysis buffer (0.05 M Tris–HCl, 0.5 M sodium chloride, 5 mM *N*-ethylmaleimide, 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl-flouride, 50 μg/ml gentamicin with protease inhibitor cocktail) and homogenized for 2 min. The solution was then kept for shaking at 4 °C for 24 h, before performing an iron assay as described earlier.

Furthermore, MRI was also performed on animals before nanoparticle injection and at the end of the study using a Varian unity INOVA 7T MR system. The multislice T2-weighted images (TR = 2500 ms; TE = 60 ms; field of view of 40 mm × 40 mm; matrix = 256 × 256; slice thickness = 1 mm) were acquired with spin echo pulse sequence. Moreover, to measure the nanoparticle concentration in the animal blood, blood (15–20 μl) was drawn from the retro-orbital sinus of the animals before nanoparticle injection and at the end of the study as described elsewhere [9]. The iron content in the blood samples was then analyzed using iron assays as described earlier.