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Salicylic Acid-Based Polymeric Contrast Agents for Molecular Magnetic Resonance Imaging of Prostate Cancer

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Supporting information and the ORCID identification number(s) for this article is given via a link at the end of the document.

Supporting Information. Synthesis of compound 3–6, characterization of the contrast agents, MR and SPECT imaging, cell and animal models and biodistribution tables. This material is available free of charge via the Internet at [http](http://).

Conflict of interest. The authors declare no conflict of interest.

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Abstract

Chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) is an innovative molecular imaging technique in which contrast agents are labeled by saturating their exchangeable protons spins by radio frequency irradiation. Salicylic acid and its analogues are a promising class of highly sensitive, diamagnetic CEST agents. We synthesized polymeric agents grafted with salicylic acid moieties and a known high-affinity ligand targeting prostate-specific membrane antigen (PSMA) in a ~10:1 molar ratio to provide sufficient MRI sensitivity and receptor specificity. We report here the proton exchange properties of the contrast agent in solution and in an experimental murine model to demonstrate the feasibility of receptor-targeted CEST MRI of prostate cancer. Furthermore, we have validated the CEST imaging data with ¹¹¹In-labeled analog of the agent by in vivo SPECT imaging and tissue biodistribution studies.

Graphical abstract

Chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) to detect tumor-specific receptor expression is not explored because of its low sensitivity. Salicylic acid-based rationally designed non-metallic polymeric contrast agent showed the feasibility of CEST MRI of prostate-specific membrane antigen (PSMA)-expression in vivo in a proof-of-concept experiment.

Keywords

receptor-targeted CEST imaging; polymeric CEST MRI contrast agent; prostate-specific membrane antigen; SPECT imaging

Introduction

Contrast agents play a significant role in clinical MRI, and are administered to detect pathology in over a third of clinical scans.^[1] Recently, a new type of MRI contrast mechanism that relies on direct chemical exchange of protons with bulk water has been developed, called chemical exchange saturation transfer (CEST) MRI.^[2] CEST MRI is an emerging molecular imaging technique in which contrast agents in low concentration are labeled by saturating their exchangeable proton spins via radio-frequency irradiation.^[3] The specificity of CEST agents is improved over relaxation based agents because of the dependence of CEST on labeling frequency.^[3–4] Furthermore, diamagnetic organic compounds, which are termed diaCEST agents, can be employed to generate contrast instead of paramagnetic metals, avoiding potential toxicity of the latter. We and others have developed salicylic acid and its analogs as diaCEST agents leveraging the far downfield chemical shift of the exchangeable proton, which allows for superior specificity of detection.^[5]

The integral membrane protein prostate-specific membrane antigen (PSMA) is an increasingly important target for both imaging and therapy of prostate cancer, particularly for the castration-resistant sub-type that claims most lives.^[6] Conventional imaging of local prostate cancer is generally performed with MRI to provide high-resolution anatomic and functional data. We have recently demonstrated that PSMA could serve as a target for MR-based molecular imaging owing to its high receptor expression within prostate cancer cells by employing a Gd^{III}-based multimeric platform.^[7] Our current goal is to develop a targeted diaCEST agent (Figure 1A) in our overall program to exploit the clinical ubiquity of MR imaging to manage prostate cancer particularly that treated with PSMA-targeted theranostics.^[6, 8]

Results and Discussion

CEST agents are largely unexplored for targeted molecular imaging mainly due to their lack of sensitivity, recently a first example was reported by us.^[9] To enhance the sensitivity of such agents for receptor-based molecular imaging in vivo, we planned to use an established polymeric platform to increase the concentration of salicylic acid moieties within a single molecular entity (Figure 1A). For this purpose, a commercially available polymeric material, poly(maleic anhydride), and its derivatives were used to graft salicylic acid units onto the multimeric backbone. These amphiphilic ‘smart’ polymer families have been extensively utilized to enhance the cytoplasmic delivery of therapeutic macromolecules in both preclinical studies and in clinical trials.^[10] Such polymeric agents offer several advantages compared to other frequently used multimeric platforms, including dendrimers and amine-based entities. Specifically, maleic anhydride and substituted copolymers are inexpensive and available in different sizes with molecular weights varying between about 10³ and 10⁶

Da. Accordingly, the biological half-life of CEST agents based on such polymers can be varied for pharmacokinetic manipulation. Also, these agents could react with free amines under mild basic condition and generate water soluble carboxylate polymers. Therefore, conjugation of chemotherapeutics or imaging agents could be performed conveniently to provide water soluble theranostic agents in high yields.

The structures of the synthesized polymeric CEST agents (**2** – **6**) investigated in this study are shown in Figure 1B. We have selected commercially available 5-(aminomethyl)-2-hydroxy-benzoic acid, **1**, in order to incorporate salicylic acid moieties onto the polymers. Compound **1** can be readily grafted onto a polymeric material functionalized with carboxylic acid or activated acid functionalities without compromising its sensitivity for CEST signal in terms of access of water to the exchangeable proton (Figure 2). The reactive amino group is placed at the R₅ position to avoid its effect on the (IntraMolecular-bonded Shifted HYdrogen) IM-SHY signal, as we previously reported.^[5b] CEST agents **2**, **3**, and **5** are untargeted while **4** and **6** are designed to target PSMA expression, respectively. As shown in Figure 2A, agent **2** was synthesized by using commercially available poly-isobutylene-maleic anhydride, comprised of 40 units of maleic acid linked linearly through an isobutylene linker, with **1** in 1:40 (polymer:**1**) molar ratio in presence of excess diisopropylethylamine (DIEA) in DMSO in one-pot at room temperature. After completion of the reaction, the product was extracted in 0.5 M sodium bicarbonate solution followed by purification by size exclusion or gel filtration to remove low-molecular-weight impurities. Agent **2** was obtained in high yield and purity and proved highly soluble in water and could be generated in gram scale by using this convenient, one-pot synthesis. The presence of salicylic acid moieties on the polymer backbone was confirmed by ¹H-NMR spectroscopy. Based on ¹H NMR, the calculated efficiency of incorporation of the salicylic acid unit onto the polymer backbone was ~ 90% (~36-40 units of salicylic acid) (supporting information Figure S1).

To rationalize further the general applicability and reproducibility of the synthetic route, we synthesized poly(ethylen-alt-maleic anhydride) with a molecular weight ~400 kDa, which contains ~3,100 units (Figure 3A). After purification, based on the ¹H NMR and size of the starting material, polymeric agent **3** was estimated to contain on average 2,700 ± 200 salicylic acid units (Figure 3B).

CEST agent **4**, was synthesized according to the scheme in Figure 2A by sequential addition of the PSMA targeting Lys-urea-Glu^[11] and **1** to a solution of poly-isobutylene-maleic anhydride in a molecular ratio of 1:4:40 (polymer:urea:**1**). Additionally, we synthesized two CEST agents, **5** and **6**, as the untargeted and targeted analogs of **2** and **4**, respectively, following a similar synthetic route. In this instance we reacted a commercially available 4-aminobutyl-DOTA chelating Lys-urea-Glu and **1** in 1:4:40 (polymer:DOTA-amine:**1**) and 1:4:4:40 (polymer:DOTA-amine:urea-amine:**1**) molar ratios, respectively. CEST agents **5** and **6** were prepared to validate in vivo pharmacokinetics of the agents through single photon emission computed tomographic (SPECT) imaging and by tissue biodistribution studies. Compounds **5** and **6** were radiolabeled with the γ -emitting radiometal ¹¹¹In(III) (half-life, 2.8 days). The molecular weights of the synthesized polymers were further

verified by gel electrophoresis as shown in Figure 2B for **2**, **4**, and **6** and size-exclusion high performance liquid chromatography (HPLC) (Supporting Information Figure S2 for **2**).

PSMA inhibitory activity (IC_{50}) of the synthesized agents was determined using a standard PSMA inhibition assay as described before^[12] and shown in Figure 2C. Inhibition constant (K_i) (95% confidence interval) values were 256 nM – 902 nM for **2**, 139.4 nM – 443.9 nM for **3**; 0.1 nM – 0.7 nM for **4**; 38.3 nM – 188.2 nM for **5** and 0.1 nM – 0.8 nM for **6**. In the same assay K_i of ZJ43^[13], a known PSMA inhibitor, was 0.6 nM – 5.5 nM. The data revealed that the binding affinities of the targeted agents **4** and **6** were significantly higher (~100 fold) compared to untargeted agents **2** and **5**.

CEST spectra for **2**, **4**, **5** and **6** are shown in Figure 4A. All agents demonstrated significant CEST contrast at 9.2 ppm as anticipated, with an additional broad signal component from 1–4 ppm. The CEST spectrum of **3** is displayed in Figure S3. A linear relationship between the contrast and the concentration of the agent was observed at low concentrations. The CEST MR imaging properties of the PSMA-targeted agent **4** are shown in Figure 2D and pH dependence in Figure 4B. The results indicate that the CEST contrast is dependent upon the concentration of the agent and is detectable below 0.3 mM. The agent displayed highest contrast at ~pH 6.8 – 7.2. Saturation transfer spectra of the agents were acquired as a function of saturation power to determine the exchange rates (k_{ex}) of the agents. The k_{ex} values of phenolic protons of all agents were in the same range ~ 2,000 s⁻¹, therefore, sensitivity of the agents is on the same order for $\omega_1 < 5.9 \mu T$.

In vivo CEST-MR images were acquired on a Bruker Biospec 11.7 T MR scanner. Male SCID/NOD mice bearing both PSMA(+) PC3 PIP and PSMA(-) PC3 flu xenografts on opposite upper flanks were injected with 125 mg/kg of **4** [number of mice (n) = 5] or **2** (n = 4) in PBS (Figure 5A). Additionally, CEST-MR imaging was performed without any contrast agent by intravenous administration of saline under similar experimental conditions using the same xenograft model as a control (Figure S4). The CEST imaging process includes three components: image collection, Water Saturation Shift Referencing (WASSR) B_0 correction, and contrast-to-signal filtering and contour leveling.^[14] Higher contrast enhancement (MTR_{asym}) between 7.8 – 10.2 ppm was observed during 25 – 120 min post-injection (Figure 5B) for the targeted CEST agent **4** in PSMA(+) PC3 PIP tumor ($1.50 \pm 0.54\%$) compared to PSMA(-) PC3 flu tumor ($0.30 \pm 0.39\%$) ($P = 0.0003$). The untargeted CEST agent **2** did not show any significant enhancement in either PSMA(+) PC3 PIP ($0.63 \pm 0.24\%$) or PSMA(-) PC3 flu tumors ($0.85 \pm 0.57\%$) during the same time frame as for the targeted agent (Figure 3C). Also, there are significantly higher values for PSMA(+)-to-PSMA(-) tumor ratios for the targeted contrast agent **4** compared to untargeted agent **2** (Figure S5, Table S1). To further check the specificity of the compound **4**, a blocking study was performed by pre-administration of ZJ43 (50 mg/kg) (Figure 6). Nearly 3-fold lowering of CEST contrast was observed within the PSMA(+) PC3 PIP tumor ($MTR_{asym} = 0.005$) compared to the unblocked experiments ($MTR_{asym} = 0.015$).

To confirm the CEST imaging results in vivo, CEST agents **5** and **6** were radiolabeled with ¹¹¹In at pH ~ 5.5 - 6 in 0.2 M sodium acetate buffer followed by purification by gel filtration to prepare ¹¹¹In-**5** and ¹¹¹In-**6** in suitable yield and purity [radiochemical yield ~ 50% and

radiochemical purity > 95%, specific activity $\sim 1.9 \text{ MBq nmol}^{-1}$ ($0.05 \text{ mCi nmol}^{-1}$). In vitro cell uptake studies revealed that $^{111}\text{In-6}$ had specific accumulation in PSMA(+) PC3 PIP cells ($14.31 \pm 1.3\%$ incubated dose/million cells) compared to PSMA(–) PC3 flu cells ($4.6 \pm 0.6\%$, incubated dose/million cells) (Figure S6).

Whole body SPECT-CT imaging were obtained for $^{111}\text{In-5}$ and $^{111}\text{In-6}$ [37 MBq (1 mCi), 50 mg/kg] are presented in Figure 5D. The targeted agent, $^{111}\text{In-6}$, displayed higher uptake in PSMA(+) PC3 PIP tumor compared to PSMA(–) PC3 flu tumor. Furthermore, high radiotracer accumulation was found in liver and kidney. Radioactivity concentration in liver was high even at 72 h post-injection for $^{111}\text{In-6}$, although the uptake in PSMA(+) PC3 PIP tumor was found much lower compared to 4 h post-injection (Figure S7). SPECT imaging studies of $^{111}\text{In-5}$ did not reveal any significant uptake in either tumor up to 24 h post-injection (Figure S8) and showed high liver and kidney uptake initially (Data not shown).

Tissue biodistribution of diaCEST agents $^{111}\text{In-5}$ and $^{111}\text{In-6}$ (dose 50 mg/kg , $n = 4$) revealed several characteristic features of PSMA-specific binding of the targeted agent, $^{111}\text{In-6}$, compared to the untargeted agent, $^{111}\text{In-5}$, (Figure 5E). First, $^{111}\text{In-6}$ showed significantly higher tumor uptake within the PSMA(+) PC3 PIP tumor compared to PSMA(–) PC3 flu tumor at all time-points. The targeted agent, $^{111}\text{In-6}$, exhibited significantly higher accumulation in PSMA(+) PC3 PIP tumor than the untargeted agent, $^{111}\text{In-5}$, during 1 – 4 h ($P < 0.001$ at 1 h and $P < 0.01$ at 2 h and 4 h). There was no significant difference for $^{111}\text{In-5}$ in PSMA(+) PC3 PIP and PSMA(–) PC3 flu tumor uptake at any time point ($P > 0.13$ at 1 h and $P > 0.19$ at 2 h and $P > 0.34$ at 4 h). Second, renal uptake for $^{111}\text{In-6}$ was significantly higher than the untargeted contrast agent $^{111}\text{In-5}$ ($P < 0.05$ at 1 h, 2 h and 4 h). Renal uptake of the radiotracers is partially due to the route of excretion of these agents as well as to specific uptake from the expression of PSMA in mouse proximal renal tubules.^[15] Third, PSMA-expressing salivary gland tissues showed significantly higher accumulation for $^{111}\text{In-6}$ compared to $^{111}\text{In-5}$ ($P < 0.001$ at 1 h and $P < 0.05$ at 4 h). Significantly, spleen uptake for both agents was high and may be due to the polymeric nature of the diaCEST agents. As shown in the Figure 3E at 1 hour post-injection there was no significant difference in the liver uptake for the untargeted $^{111}\text{In-5}$ ($53.72 \pm 2.63\%$ injected dose (ID)/g) compared to the targeted compound $^{111}\text{In-6}$ ($56.82 \pm 4.47\%$ ID/g) whereas at 4 h post-administration, targeted $^{111}\text{In-6}$ ($37.34 \pm 8.97\%$ ID/g) displayed a significant clearance ($P < 0.05$) from the liver compared to the untargeted $^{111}\text{In-5}$ ($62.94 \pm 2.96\%$ ID/g). Detailed biodistribution results of sixteen tissues are included in Tables S2 and S3.

The salicylic acid-based targeted contrast agent, compound **4**, has two major limitations for near-term clinical translation. First is its low CEST-MR imaging sensitivity. That was the reason we synthesized compound **3** ($\sim 1,000 \text{ kDa}$). However, based on preliminary experiments, we realized that such a large polymer would also generate high nonspecific signal from the PSMA(–) tumor due to the enhanced permeability and retention effect and also would be sequestered by normal organs including liver and spleen. In fact, high liver and spleen uptake is also the major concern with compound **4** ($\sim 12 \text{ kDa}$). Intravenous injection of a compound with a polymeric backbone with ~ 40 carboxylic acids, as in compound **4**, will still engender opsonization and sequestration by the mononuclear

phagocytic system within liver and spleen and provide only a modest amount of PSMA-specific binding. The most common strategy to avoid nonspecific protein binding and phagocyte uptake is to engraft the polymers with neutrally charged linear chains of poly(ethylene glycol) (PEG).^[16] Accordingly, our current focus is to modify compound **4** with an optimized number of PEG units (MW 1 – 5 kDa) to reduce the overall surface charge and also to increase circulation time. We also anticipate that the next generation compounds with targeting moieties attached to optimized PEG linkers might also allow higher access to receptor binding to provide higher tumor uptake and internalization within the PSMA(+) tumor compared to compound **4**.

While radionuclide imaging (SPECT/PET) has its own advantages, specifically the sensitivity of the modality for receptor-based imaging, diaCEST-based imaging also has a variety of advantages. These include the availability of a ubiquitous clinical modality (MRI) with high spatial resolution and exquisite soft-tissue delineation, no use of ionizing radiation, lower cost (¹¹¹In is an expensive isotope, on the order of thousands of dollars per synthesis), and potential to discriminate multiple contrast agents through their different resonant frequencies. These are considerable advantages for targeted imaging studies.

Conclusions

We have synthesized and characterized a new class of polymeric CEST contrast agents to detect PSMA expression *in vivo* for molecular MR imaging of prostate cancer. The polymeric materials employed here could be used as a platform for targeted CEST imaging for a wide variety of medically important receptors, enzymes and transporters.

Experimental Section

General Experimental Method

Solvents and chemicals purchased from commercial sources were of analytical grade or better and used without further purification. 5-Methyl amino-2-hydroxybenzoic acid, diisopropylethylamine (DIEA), triethylamine (TEA) and the polymer (poly(isobutylene-alt-maleic anhydride)₄₀, MW ~6 kD) were purchased from Sigma-Aldrich. Aminobutyl-DOTA was purchased from Macrocyclics, Inc. (Dallas, TX). [¹¹¹In]InCl₃ was obtained from Nordion, Canada. The polymer poly(ethylen-*alt*-maleic anhydride)₃₁₀₀ was purchased from PolyScience (<https://www.polyscience.com>). Flash chromatography was performed using silica gel (MP SiliTech 32-63 D 60Å) purchased from Bodman (Aston, PA). Compounds Glu-Lys-urea amine was synthesized following our previous report.^[11] All other chemicals were purchased from Thermo Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

All *in vitro* PSMA binding studies were performed in triplicate to ensure reproducibility, as previously reported.^[12] ¹H NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts (δ) are reported in ppm downfield in reference to proton resonances resulting from incomplete deuteration of the NMR solvent. Quantitative ¹H NMR was used to prove that all synthesized compounds were at > 90% chemical purity. *In vivo* CEST-MR images were acquired on a Bruker Biospec 11.7 T MR scanner.

Synthesis of the Polymeric Contrast Agents

Compound 2—The polymer (poly(isobutylene-alt-maleic anhydride)₄₀, MW ~6 kD) (100 mg, 16.67 μ mol), and compound **1**, 5-methyl amino-2-hydroxybenzoic acid, (135 mg, 0.67 mmol) were dissolved together in 2 mL DMSO in a glass vial, followed by addition of 290 μ L of DIEA (1.6 mmol) to produce a colorless solution. The solution was left stirring overnight (~ 20 h) at room temperature. The solvent was removed under reduced pressure and the colorless residue was dissolved in 3 X 10 mL 0.5 M NaHCO₃ and was spin filtered using 3 K MWCO filter. The filtrate was further washed water (5 X 10 mL) or until the pH of the filtrate decreased to ~ 7.5. The filtrate was lyophilized to generate a colorless solid. Yield: ~ 100 mg. δ ppm 0.66 – 0.98 (m, 7H) 1.63-2.64 (m, 4 H), 3.22-4.14 (m, 2H) 6.72 - 7.67 (m, 3 H).

Synthesis of Compound **3-6** were performed same general method as described for compound **2** and provided in the Supporting Information.

Radiolabeling Methods—¹¹¹In-Labeling of contrast agents **5** and **6** were performed according to our previously reported method.^[12, 17] Briefly, a solution of ¹¹¹InCl₃ (81.4 MBq (2.2 mCi)/5 μ L) was added to solution of 0.2 M 90 μ L solution of sodium acetate at pH ~ 4.5 followed the addition of 10 μ L of either **5** or **6** (2 mM solution in water) in a 1.5 mL polypropylene vial and was kept at 50°C for 1 h. A solution of disodium-EDTA (10 μ L, 10 mM) was added to reaction vial and kept for 5 min at room temperature to quench unreacted ¹¹¹InCl₃. After cooling the reaction mixture to room temperature, polymeric contrast agents were purified using a Zeba Spin column (7K MWCO), pre-equilibrated with 0.9 % saline. Filtrate was collected and purified four times using four separate columns to remove any unreacted ¹¹¹In to ensure high purity. Yield ~ 37 MBq (1 mCi) (~50 %), specific activity ~ 1.9 MBq/nmol (~ 0.05 mCi/nmol). For all in vitro and in vivo experiments (biodistribution and imaging studies) dose preparation was done using 0.9 % saline.

PSMA Inhibition Assay—The PSMA inhibitory activity of **2**, **4**, **5**, and **6** were determined using a fluorescence-based assay according to a previously reported procedure.^[12] Inhibition curves were determined using semi-log plots and IC₅₀ values were determined at the concentration at which enzyme activity was inhibited by 50 %. Enzyme inhibitory constants (K_i values) were generated using the Cheng-Prusoff conversion.^[18] Assays were performed in triplicate. Data analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California).

SDS-polyacrylamide Gel Electrophoresis—20 μ g of the compounds were loaded with 1 $\times$ LDS sample buffer (Novex, NP0007) on 10% Bis-Tris SDS-polyacrylamide gel (Novex, NP0301BOX) and was electrophoresed on constant 200V for 40 min. After the electrophoresis, the compound bands were visualized by UV illumination and image was captured.

Cell Lines—Sublines of the androgen-independent PC3 human prostate cancer cell line, originally derived from an advanced androgen independent bone metastasis, were used. Those sublines have been modified to express high levels of PSMA [PSMA-positive (+) PC3

PIP] or are devoid of target [PSMA-negative (–) PC3 flu]. They were generously provided by Dr. Warren Heston (Cleveland Clinic).

Tumor Models—Animal studies were undertaken in compliance with the regulations of the Johns Hopkins University Animal Care and Use Committee. Six- to eight-week-old male, Nonobese Diabetic (NOD)/Severe Combined immunodeficient (SCID) mice (Johns Hopkins Immune Compromised Animal Core) were implanted subcutaneously (sc) with PSMA(+) PC3 PIP and PSMA(–) PC3 flu cells (1×10^6 in 100 μ L of HBSS (Corning Cellgro, Manassas, VA) at the backward right and left flanks, respectively. Mice were imaged or used in biodistribution assays when the xenografts reached 5 to 7 mm in diameter.

MR Imaging Methods—Phantom preparation and in vitro CEST MRI data acquisition: Polymer samples were dissolved in 0.01 M phosphate-buffered saline (PBS) at concentrations from 0.3 mM to 2.5 mM, and titrated using high-concentration HCl/NaOH to various pH values ranging from 6 to 8. The solutions were placed into 1 mm glass capillaries and assembled in a holder for CEST MR imaging. The samples were kept at 37 °C during imaging. Phantom CEST experiments were collected on a Bruker 11.7 T vertical bore MR scanner, using a 20 mm birdcage transmit/receive coil. CEST images were produced using a RARE (RARE = 12) sequence with a CW saturation pulse length of 3.5s and saturation field strengths (B_1) varying from 1.2 μ T to 7.2 μ T. The Z-spectra (saturation spectra as a function of saturation frequency referenced to water at 0ppm) were acquired by incrementing the saturation frequency every 0.25 ppm from –15 to 15 ppm for phantoms; TR = 7 s, effective TE = 26 ms, matrix size = 64×48 and slice thickness = 1 mm following the previously published methods.^[3b, 19]

In vivo MRI and data processing—In vivo images were collected on a Bruker Biospec 11.7 T horizontal bore MR scanner, using a 23 mm transmit/receive mouse coil. The tumor-bearing NOD/SCID mice ($n = 4$ for both the targeted polymer, contrast agent **4**, and the untargeted polymer, contrast agent **2**) were anesthetized by inhaling 0.5–2 % isoflurane with breath rate monitored throughout the MRI session using a respiratory probe. CEST images were repeatedly collected with 15 saturation offsets (from 7.2 ppm to 11.4 ppm with 0.3 ppm increment) both pre- and post-injection for one axial slice 1.2 mm thick displaying the middle of both tumors. Image parameters were similar to those used for phantoms except for TR/TE=5 s/15 ms, saturation length 2 s, $B_1 = 5.9 \mu$ T and spatial resolution: $350 \mu\text{m} \times 350 \mu\text{m}$. A 100 μ L volume of 25 mg/ml polymer solution in PBS ($\sim$ pH 7) was slowly injected by a catheter into the tail vein. CEST contrast was quantified by $\text{MTR}_{\text{asym}} = (S_{-\omega} - S_{+\omega})/S_{-\omega}$ where $S_{-\omega}$ and $S_{+\omega}$ refer to the water signal intensity with a saturation pulse applied at the frequencies $-\omega$ and $+\omega$ with respect to the water proton resonance frequency, respectively and was integrated over the range of 7.8 – 10.2 ppm based on the MTR_{asym} curves in phantoms shown in Fig. 2 to maximize the contrast-to-noise ratio of the in vivo images.

Blocking Experiment for compound 4—A pre-blocking experiment was performed using ZJ43 (50 mg/kg), a known PSMA inhibitor that uses the identical PSMA targeting moiety Lys-urea-Glu as in compound **4**. The blocking agent was administrated 15 min

before the contrast agent administration. Compound **4** demonstrated significant lowering (3-fold) of CEST contrast within the PSMA(+) PC3 PIP tumor ($MTR_{\text{asym}} = 0.005$).

Cell up take Study—Cell uptake study was performed following an established method reported by us.^[20] Briefly, PSMA-targeted polymer, **¹¹¹In-6** and untargeted polymer **¹¹¹In-5** [37 kBq (1 μ Ci)] ($n = 4$) were incubated in PSMA(+) PC3 PIP and PSMA(−) PC3 flu cells (1×10^6) at 37°C for 2 h followed by washing with PBS.

In Vivo SPECT-CT Imaging—Two mice each bearing PSMA(+) PC3 PIP and PSMA(−) PC3 flu dual xenografts were injected intravenously with 37.0 ± 3.7 MBq (1 mCi $\pm$ 100 μ Ci) of either **¹¹¹In-5** or **¹¹¹In-6** in physiological saline and images were acquired on X-SPECT (Gamma Medica-Ideas, Inc., Northridge, CA). The SPECT data were reconstructed and co-registered with CT using the manufacturer's software and displayed using AMIDE (www.amide.sourceforge.net).

Biodistribution—Biodistribution study was performed following our previous report.^[12] Mice bearing PSMA(+) PC3 PIP and PSMA(−) PC3 flu xenografts were injected *via* the tail vein with 1.85 MBq (50 μ Ci) of either **¹¹¹In-5** or **¹¹¹In-6** in 150 μ L of saline ($n = 4$ per time-point). The percentage of injected dose per gram of tissue (% ID/g) was calculated by comparison with samples of a standard dilution of the initial dose. Detail tissue biodistribution data are included in Table S2 and Table S3.

Data Analysis—Data are expressed as mean $\pm$ standard error of the mean (SEM). Prism software (GraphPAD, San Diego, California) was used to determine statistical significance. The animal model we have used, serves as both positive and negative control because of the fact that each animal bears both PSMA(+) and PSMA(−) tumors. Accordingly a paired t-test was used to check the differences in the contrast (MTR_{asym}) generated by the PSMA-targeted CEST agents (compound **4** and compound **2**). Our hypothesis is that for each animal, the ratio of MTR_{asym} for PSMA(+)-to-PSMA(−) will be > 1 . Additionally a two sample (since **4** vs **2**) unpaired t-test was performed using the ratios of MTR_{asym} of PSMA(+)-to-PSMA(−) tumors to evaluate statistical significance of the targeted vs. untargeted contrast compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Salicylic acid-based diaCEST agents. A. General concept. B. CEST agents **2**, **3** and **5** are untargeted while **4** and **6** target PSMA through the Lys-urea-Glu moiety (blue).

Figure 2.

A. Synthetic scheme for polymeric CEST **2** and **4**. B. Gel-electrophoresis indicating the molecular weight of the CEST agents at ~10 kDa by UV illumination. C. PSMA-inhibitory activity (IC₅₀ curves) of **2**, **4**, **5** and **6**. D. CEST spectra of **4** in PBS at several concentrations.

Figure 3.

A. Synthesis of **3** Conditions: a) DIEA/DMSO; b) spin filtration (3 MWCO with 0.5 M NaHCO₃ and then water). **B.** ¹H-NMR spectrum of contrast agent **3** in D₂O at room temperature.

Figure 4.

A. CEST spectra of the contrast agents 2, 4, 5 and 6 (0.5 mM) at pH 7.3 - 7.4. **B.** CEST spectra of the contrast agent 4 (2.5 mM) at ~pH 6 - 7.85 at 5.9 μ T. All CEST data were obtained $t_{\text{sat}} = 2$ sec $B_1 = 5.9$ μ T and $T = 37^\circ\text{C}$.

Figure 5.

(A) In vivo CEST imaging of salicylic acid-based polymeric diaCEST agents **2** (untargeted) and **4** (PSMA-targeted). Mice bearing two human prostate cancer xenografts [PSMA(+) PC3 PIP and PSMA(-) PC3] in opposite flanks were injected intravenously with 125 mg/kg of either **2** or **4** in PBS. Fifteen offsets were collected from 7.2 ppm to 11.8 ppm with CEST imaging parameters: $t_{\text{sat}} = 2$ s, $B_1 = 5.9$ μ T, spatial resolution: $350 \mu\text{m} \times 350 \mu\text{m}$ and the contrast $\text{MTR}_{\text{asym}} = \text{MTR}_{\text{asym}}(t) - \text{MTR}_{\text{asym}}(t = 0)$ integrated over this spectral range; During 25 – 120 min post-injection significant contrast was observed for **4** (upper panels) in the PSMA(+) PC3 PIP tumors, but not in the control, PSMA(-) PC3 flu tumor; Compound **2**, the untargeted polymer (lower panels) shows no contrast enhancement. (B) CEST contrast as a function of time from regions of interests (ROIs) defined in A for the targeted contrast agent **4**, PSMA(+) PC3 PIP tumor (red solid line) and PSMA(-) PC3 flu tumor (red dotted line). (C) Mean changes in CEST signal as quantified by MTR_{asym} (90 – 110 min) in each tumor type [PSMA(+) PC3 tumor (solid line), PSMA(-) PC3 flu tumor (dotted line)] [$n = 4$ for **2** (blue); $P = 0.5$ and $n = 5$ for **4** (red); $P = 0.0003$] within the saturation frequency range 7.8 – 10.2 ppm, (Student's t test, two-tailed and paired); Notice the broadened curve, which we ascribe to be a consequence of the well-known lower pH in the extravascular extracellular space of tumors. (D) SPECT/CT ($n = 2$) images of PSMA-targeted polymer ^{111}In -**6** and untargeted polymer ^{111}In -**5** at 90 – 120 min post-injection using a dose of 37 MBq (1 mCi) (50 mg/kg, i.v.). (E) Tissue biodistribution ^{111}In -**6** and ^{111}In -**5** ($n = 4$) at 1 and 4 h post-injection employing the same animal model as used for imaging studies. B, blood, H, heart, L, Liver; K, kidney, M, muscle, S, spleen, PIP, PSMA(+) PC3 PIP tumor, flu, PSMA(-) PC3 flu tumor. $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***)

Figure 6. Blocking agent ZJ43 (50 mg/kg) was administered 15 min prior the CEST contrast agent administration. Significant lowering of the CEST contrast signal was observed in the PSMA(+) PC3 PIP tumor (B) compared to unblocked experiment (A).