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Ultra-High-Frequency-Radio-Frequency-Acoustic Molecular Imaging with Saline Nanodroplets in Living Subjects

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Abstract

Molecular imaging is a crucial technique in clinical diagnostics, but it relies on radioactive tracers or high magnetic fields that are unfavorable for many patients, particularly infants and pregnant women. Ultra-high-frequency-radiofrequency-acoustic (UHF-RF-acoustic) imaging using non-ionizing RF pulses allows deep-tissue imaging with sub-millimeter spatial resolution. However, lack of biocompatible and targetable contrast agents has prevented the successful in vivo application of UHF-RF-acoustic imaging. Here, we report our development of targetable nanodroplets for UHF-RF-acoustic molecular imaging of cancers. We synthesize all-liquid nanodroplets containing hypertonic saline that are stable for at least 2 weeks and can produce high intensity UHF-RF-acoustic signals. Compared with concentration-matched iron-oxide nanoparticles, our nanodroplets produce at least 1600 times higher UHF-RF-acoustic signals at the same imaging depth. We demonstrate in vivo imaging using the targeted nanodroplets in

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AUTHOR CONTRIBUTIONS

SSG conceived the original idea. Y-SC and SSG designed the experiments. Y-SC developed the contrast agents and performed the in vitro and in vivo imaging. Y-SC, YZ, and HW performed the theoretical study. DHC contributed to cryo-EM. CB, AZ, ECH, TS, and JAD contributed to the discussion of the data and experimental results. Y-SC and SSG drafted the manuscript and all authors contributed to the writing of the manuscript. SSG supervised the entire study.

COMPETING INTERESTS

SSG declares competing financial interests with Endra Inc. and Visualsonics Inc.

DATA AVAILABILITY

The data that support the plots within this paper and other findings of this study are available from the corresponding author upon reasonable request.

ADDITIONAL INFORMATION

Supplementary information is available in the online version of the paper. Reprints and permission information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to Y-SC.

a prostate cancer xenograft mouse model expressing gastrin release protein receptor (GRPR), showing targeting specificity by more than two-fold, compared to untargeted nanodroplets or prostate cancer cells not expressing GRPR.

Keywords

RF-acoustic imaging; molecular imaging; nanodroplets; contrast agents; prostate cancer

Early cancer diagnosis remains one of the top aims in clinical oncology since it has been shown to greatly improve treatment effectiveness and disease containment¹. To detect cancers noninvasively at an early stage, high sensitivity, high spatial resolution, and large imaging depth are often needed simultaneously^{2, 3}. Unfortunately, enhancing one capability often leads to tradeoffs with the other. For example, positron emission tomography (PET) and single-photon emission computed tomography (SPECT) offer high sensitivity with infinite imaging depth but provide moderate imaging resolution. Computed tomography (CT) and magnetic resonance imaging (MRI) provide millimeter spatial resolution with relatively low molecular sensitivity^{3–7}. Moreover, PET, SPECT and CT all use ionizing radiation and are not the valid choice for patients vulnerable to radiation exposure⁸. Molecular ultrasound imaging uses non-ionizing sound waves to image and provides high spatial resolution down to micrometers, but the large size of imaging agents (microbubbles) suffers from the non-extravasation to the tumor site^{9, 10}. Several nano-sized ultrasound imaging agents, such as acoustic nanodroplets, have been developed recently¹¹. However, they usually require an external ultrasound burst to vaporize the nanodroplets to microbubbles, thus may limit the targets to where the proximal positions are known¹¹. On the other hand, molecular photoacoustic imaging accommodates extravasated contrast agents, but they typically penetrate shallower than 3 cm in tissue due to strong attenuation of light¹². As such, developing new non-ionizing molecular imaging techniques towards imaging at high depths and spatial resolution with high sensitivity remains an outstanding challenge.

Radio-frequency-acoustic (RF-acoustic) imaging is an emerging technique that generates ultrasound images by illuminating tissue with non-ionizing electromagnetic pulses in the frequency range of 20 kHz–300 GHz (Figure 1a)^{13–19}. In tissue, electromagnetic waves decay with depth due to absorption and scattering, where the decay rate is frequency dependent. RF waves in the ultra-high-frequency (UHF) band (300 MHz – 1 GHz) decay 100 times slower with depth than light, and 10 times slower than microwave (1 GHz – 300 GHz)²⁰. Thus, UHF-RF-acoustic (Figure 1b) can potentially image much deeper compared to optical, photoacoustic, or microwave-acoustic imaging techniques (see ‘Comparisons of UHF-RF-acoustic Imaging with Other Acoustic-based Imaging’ in Supplementary Information)^{19, 21}. In addition, differences in RF-absorption between cancerous and normal tissue in the UHF range have been reported, suggesting that UHF-RF-acoustic imaging can potentially differentiate cancerous tissue from healthy tissue^{11, 12}. However, due to the limited choices of biocompatible UHF-RF absorbing materials to date, the application of UHF-RF-acoustic molecular imaging using exogenous contrast agents in vivo has not been

reported. In this work, we report the development and thorough evaluation of a stable liquid UHF-RF-acoustic nanoparticle, and its utility for molecular imaging of prostate cancer.

Design Concept for Achieving UHF-RF-acoustic Contrast

In UHF, Joule heating caused by collision of charged carriers is the dominant heating mechanism²⁰. Ideal choices for UHF-RF-acoustic contrast agents are materials that either have high dielectric loss or conductivity at the frequency of interest (300 MHz-1 GHz)^{20, 22}. While several types of nanoparticles such as gold, silicon, carbon nanotube, and iron oxide nanoparticles have been investigated for RF-acoustic imaging, these particles showed weak RF-acoustic signals in the UHF range^{20, 22–24}. On the other hand, liquid electrolytes with high ionic conductivity, such as aqueous salt solutions produce much stronger UHF-RF-acoustic signals, making them ideal choices for developing UHF-RF-acoustic contrast agents^{22, 25}. To determine the ideal salt solution, five candidates, NaCl_(aq), KI_(aq), KCl_(aq), MgCl_{2(aq)}, and CaCl_{2(aq)}, were assessed for their ionic conductivity, solubility in water, biocompatibility, and UHF-RF-acoustic signals. In addition, NaOH_(aq) was also used as a positive control due to its known high ionic conductivity albeit its toxicity. The UHF-RF-acoustic signals were measured using a prototype RF-acoustic tomography system (see ‘UHF-RF-acoustic Imaging System’ in Supplementary Information) with nanosecond UHF-RF pulses at 433 MHz frequency, which is within the medical device radio-communications service band (MedRadio) (Figure 1).

As expected, Figure 2b shows that NaOH_(aq) (4 wt%, to avoid signal saturation) produces the strongest UHF-RF-acoustic signal, followed by KCl_(aq) and NaCl_(aq) (both 10 wt%). Interestingly, linear correlation between the UHF-RF-acoustic amplitude of these electrolyte solutions and their conductivity was found ($R^2 = 0.92$), confirming that the UHF-RF-acoustic signals are mainly related to the conductivity and minimally associated with the type of salt (Figure 2c). Since saline is shown to have the highest electrical conductivity among these salts at its saturated concentration (~25 wt%)^{26, 27}, we decided to further investigate its potential as an UHF-RF-acoustic contrast agent.

To provide molecular information using UHF-RF-acoustic imaging, the contrast agent needs to be targeted to a specific marker of disease. Unfortunately, salts in water consist of ions which easily diffuse away in tissue and cannot be functionalized to molecular targeting ligands, such as antibodies and peptides. Hence, saline needs to be first encapsulated into a stable nanoparticle for subsequent conjugation with molecularly targeted ligands. Many approaches using hydrophobic biomaterials (such as lipids and biodegradable polymers) to encapsulate aqueous nanodroplets (also called double emulsions, or water/oil/water (W/O/W) emulsions) have been used in medicine^{28, 29}, cosmetics and food industry^{30, 31}, as well as imaging^{32, 33}. Unfortunately, none of these existing nanodroplets can stably sustain the large outward osmotic pressure caused by the differential ionic concentrations between the core and outer aqueous solutions^{34, 35}. Such stability, however, is essential for producing reliable imaging signals for medical imaging applications.

Previous theoretical studies showed that altering solute and water permeability of the oil phase greatly affects the size of the nanodroplets³⁶, suggesting that reducing water

permeability in the oil phase can significantly reduce its swelling^{36–38}. Macroscopically, water permeability is directly related to bulk water solubility of the material. After reviewing water solubility in various biocompatible liquids^{35, 39, 40}, perfluorocarbon liquids were chosen for our nanoparticle formulation due to their significantly lower water solubility and frequent utility in various biomedical applications⁴¹. Perfluorocarbon liquids, including perfluoropentane⁴², perfluorohexane⁴², perfluoro-15-crown-5-ether⁴², and perfluorodecalin⁴³ were evaluated to determine the optimum shell to stabilize our saline droplets. In addition, soybean oil⁴⁴, a commonly used hydrocarbon liquid, was used as a reference “oil phase”.

Synthesis and Stability Characterization of Saline Nanodroplets

A double emulsion method was developed to encapsulate hypertonic saline (up to 25 wt%) inside the nanoparticles (Figure 2d, ‘Synthesis of Nanodroplets’ in Supplementary Information). To confirm saline encapsulation, the double-emulsion nanodroplets were compared with the single-phase perfluorohexane nanodroplets using cryo-electron microscopy. Figure 2e shows the differential contrasts inside the double-emulsion, confirming the core-shell configuration (left panel); whereas uniform contrast of the single-phase nanodroplets was observed (right panel). In addition, we were able to control the size of the nanodroplets and produce uniform particles (polydispersity < 0.1) by adjusting the ultrasound power and the pore size of the extrusion filter (Figure 2f, and Supplementary Figure S2).

The stability of the nanodroplets was monitored for two weeks by assessing changes in size using dynamic light scattering (DLS). Figure 2g shows that the soybean oil nanodroplets more than doubled in size (~215%) after only 6 days, whereas negligible changes on the perfluorocarbon nanodroplets were observed. After 6 days, due to the large polydispersity of the soybean oil nanodroplets, we could no longer measure their sizes using DLS, while the size of perfluorocarbon nanodroplets remained stable for up to 14 days. Specifically, the increase in diameters of perfluorodecalin, perfluoro-15-crown-5-ether, perfluorohexane, and perfluoropentane nanodroplets were $18 \pm 15\%$, $28 \pm 17\%$, $65 \pm 17\%$, and $103 \pm 18\%$ after 14 days, respectively. These results indicate the importance of the utilized perfluorocarbon liquids and their effects on nanodroplet stability.

It is known that stability of perfluorocarbon nanodroplets are strongly related with molecular diffusivity of perfluorocarbons, which is greatly affected by the molecular weight and the structure of the perfluorocarbon molecules⁴⁵. Typically, the decrease in molecular diffusivity results in decrease of water solubility, increase of interfacial tension, and slowing of the rate of Ostwald ripening, and ultimately increase of the emulsion stability^{38, 46}. In the nanodroplets (double emulsions), the diffusivities could also affect the rates of inward water permeability and hence stability. The different types of perfluorocarbon nanodroplets have different shelf-lives in our stability study, likely due to their differences in molecular diffusivity (Figure 2g). While it is non-trivial to directly measure the molecular diffusivities of the perfluorocarbon molecules in nanodroplets, a strong relation between the molecular diffusivity and the vapor pressure has been previously demonstrated³⁸. Specifically, high vapor pressure liquid has a high molecular diffusivity because of the weak interactions

between molecules⁴⁷; therefore, vapor pressure of each perfluorocarbon liquid is a more predictive factor than molecular diffusivity to test the stability of nanodroplets. From Figure 2g and Supplementary Table S2, it shows size expansion of nanodroplets decreases as the vapor pressure of perfluorocarbon liquids decreases.

To further examine how vapor pressure alone affects the stability, we vary the vapor pressure of perfluorocarbon within the same type of perfluorocarbon nanodroplets by changing their sizes. When the surfactants and surrounding solvent are the same, it is known that the vapor pressure of perfluorocarbon liquid in the emulsion is mainly affected by the size-dependent Laplace pressure, which is a work balance between tension and applied pressure at the curved interface of particles^{42, 47, 48}. When the size of nanodroplets decreases, Laplace pressure increases and leads to a high vapor pressure. Similar effects have been demonstrated in the phase-changing-perfluorocarbon single emulsions, in which perfluorocarbon nanodroplets can be vaporized from liquid nanodroplets to gas microbubbles by ultrasound exposure^{42, 49}. It has been shown that small nanodroplets require a higher energy to be vaporized because the Laplace pressure increases the vapor pressure, causing the increase of boiling point of the perfluorocarbon in the nanodroplets^{42, 49}. To test this hypothesis, we developed two larger nanodroplets (~450 nm and ~800 nm in average diameter) aiming to decrease the Laplace pressure and consequently reduce the vapor pressure of perfluorocarbon droplets. We used perfluoropentane since its boiling point is close to room temperature (28 °C), which is at the boundary of becoming unstable and more susceptible to the increment of size. Figure 2h confirms that among the perfluoropentane nanodroplets, greater increase in sizes (i.e. reduced stability) of the larger nanodroplets were observed, compared to the small nanodroplets upon incubation for 14 days ($103 \pm 18\%$, $139 \pm 18\%$, and $142 \pm 20\%$ for 250-nm, 450-nm, and 800-nm respectively). Overall, these results suggest that choosing a perfluorocarbon liquid with a high vapor pressure or reducing the size of the nanodroplets can improve the stability for longitudinal imaging.

Optimization of UHF-RF-acoustic Signals

To determine the optimum saline concentration, nanodroplets encapsulated with four concentrations of NaCl_(aq) solutions (25 wt%, 20 wt%, 15 wt%, and 10 wt%) at 2×10^9 nanodroplets/mL were assessed in a tube phantom (Figure 3a–d, Supplementary Figure S3). As a control, another tube was filled with physiological saline (0.9 wt% of NaCl) to mimic UHF-RF-acoustic background signals of tissue. As expected, the UHF-RF-acoustic signal of the nanodroplets increases linearly with the saline concentration ($R^2 = 0.99$), confirming the successful encapsulation of saline within these nanodroplets. We then selected the optimum nanodroplets with 25 wt% saline and assessed the correlation between UHF-RF-acoustic signals and nanodroplets concentrations. Figure 3b shows that the UHF-RF-acoustic signals highly correlate with the concentration of the nanodroplets ($R^2 = 0.99$). In addition, 2×10^8 nanodroplets/mL (25 wt%) and physiological saline (no droplets) produce comparable UHF-RF-acoustic signals, providing an estimated detection limit of these nanodroplets in tissue.

We compared the performance of our nanodroplets with other nanoparticles operating in the RF range but at higher frequencies (microwave, >1 GHz)^{24, 49} using a similar tube phantom

(Figure 2a). Four tubes were filled with nanodroplets (25 wt% saline, 1×10^9 nanodroplets/mL), one with gold nanoparticles (20 nm in diameter, 5×10^{11} nanoparticles/mL), and one with iron oxide (Fe_3O_4) nanoparticles (100 nm in diameter, 5×10^{11} nanoparticles/mL). Figure 3d shows the UHF-RF-acoustic imaging of these tube phantoms (middle panel) and the statistical comparison of their quantified signals (right panel). Although the gold and Fe_3O_4 nanoparticles were 500 times more concentrated than our nanodroplets solution, UHF-RF-acoustic signals from our nanodroplets were statistically higher. We quantified the signal amplitude from the images and normalized it by the nanoparticle concentration. The result shows the UHF-RF-acoustic signal of our nanodroplets is 2500 ± 30 and 1600 ± 35 times higher than gold and Fe_3O_4 nanoparticles.

One of the key strengths of UHF-RF-acoustic molecular imaging is its potential in deep tissue imaging applications. To demonstrate this capability, we built two bovine tissue phantoms. One phantom contains six 3-mm-diameter inclusions at different depths beneath the surface (Figure 3e). Inclusions located 5 cm deep in tissue were detectable. For demonstration in even deeper tissue, another bovine tissue phantom ($\sim 9 \text{ cm} \times 9 \text{ cm}$) was developed, where a hole (2.2 cm in diameter) was drilled in the tissue, and tightly fitted with a Stanford logo-shaped agar gel phantom (Figure 3f). The logo contains spatial features as small as $\sim 1\text{--}2 \text{ mm}$ (the branches of the redwood tree), with which we demonstrated deep-tissue imaging of 25 wt% saline nanodroplets at 7 cm beneath the surface, visualizing the 1-mm features (Figure 3f right and Supplementary Figure S5).

In Vivo UHF-RF-acoustic Molecular Imaging

Gastrin-releasing peptide receptor (GRPR) is our target of interest for in vivo molecular imaging, as it is a biomarker overexpressed in many cancers, including prostate, breast, colon, and lung cancers²⁵. Targeting to GRPR was achieved by coupling an anti-GRPR antibody to the saline nanodroplet surface; a maleimide-ICG dye was also linked to the surface to allow fluorescence imaging for assessing targeting specificity in vitro and in vivo (see ‘Anti-GRPR Antibody and ICG Dye Conjugation on Nanodroplets’ in Supplementary Information).

We first confirmed GRPR expression in a panel of prostate cancer cell lines using western blot analysis (Figure 4b). High GRPR-expressing PC3 cells (GRPR⁺) and low GRPR-expressing DU145 cells (GRPR⁻) were chosen as our positive and negative controls, respectively. Before the in vitro binding studies, cytotoxicity of the targeted nanodroplets on prostate cancer cells was evaluated using Presto Blue cell viability assay. Our results show no obvious reduction in cell viability when cells were incubated with up to 1×10^{11} nanodroplets/mL for 24 hours, while a reduction to 70% with 3×10^{11} nanodroplets/mL (Figure 4c).

To evaluate GRPR-targeting specificity of our nanodroplets, both GRPR⁺ and GRPR⁻ cell lines were incubated with either targeted (GRPR) or non-targeted (PEG) nanodroplets (1×10^8 nanodroplets for 1×10^5 cells) for two hours. PC3 cells show 2.5-fold higher ICG fluorescence signals when incubated with GRPR-targeted nanodroplets compared to non-targeted nanodroplets (Supplementary Figure S8). As expected, the negative control cell line DU145 shows negligible fluorescence signals when incubated with either targeted or

non-targeted nanodroplets (Figure 4d and Supplementary Figure S8). To further confirm molecular specificity, we performed a blocking study where PC3 cells were pre-incubated with anti-GRPR antibody in excess for 30 minutes prior to incubation with GRPR-targeted saline nanodroplets. Indeed, the pre-incubation with anti-GRPR antibody significantly decreased the binding of the nanodroplets, as shown by the lower ICG fluorescence signals compared to that without blocking (Figure 4d). Overall, our in vitro blocking tests (Supplementary Figure S8) and competitive binding experiments (Supplementary Figure S9) confirm the specificity of our GRPR-targeted saline nanodroplets.

Six-weeks-old male NOD-SCID-IL2R γ^{null} (NSG) tumor-bearing mice were randomized into four groups (N=5 per group): two groups of mice were subcutaneously implanted with PC3 cancer cells (groups 1, 3) and two groups were implanted with DU145 cells (groups 2, 4) at the middorsal region of each mouse (see 'Animal Studies' in Supplementary Information). In addition to UHF-RF-acoustic imaging, the pharmacokinetics, in vivo targeting specificity and biodistribution of ICG-labeled nanodroplets were also evaluated by fluorescence imaging using ICG-nanodroplets (Supplementary Figures S13–S15). Each tumor-bearing mouse was injected with 100 μL of nanodroplets solution via the tail vein (1×10^{11} nanodroplets/mL), where groups 1 and 2 were injected with GRPR-targeted nanodroplets; while groups 3 and 4 were injected with non-targeted nanodroplets. Forty-eight hours after injection, UHF-RF-acoustic and fluorescence imaging of all mice were performed. A representative mouse image from each group was shown in Figure 5b, showing visibly higher UHF-RF-acoustic signals from the PC3 tumor with GRPR-targeted nanodroplets (group 1), compared to tumors in groups 2–4, indicating the in vivo GRPR-specificity of our targeted nanodroplets. Quantitative analyses (Figure 5c) of the UHF-RF-acoustic signals also show significantly higher signals in group 1 (2.4 ± 0.3 -fold) than groups 2–4. Immediately after UHF-RF-acoustic imaging, whole body fluorescence imaging was performed to confirm the successful delivery and targeting of the ICG-labeled nanodroplets (Figure 5d). The higher ICG fluorescence signals in the tumors of group 1 compared to groups 2–4 further confirmed the specificity of the targeted nanodroplets to GRPR-expressing tumors, which corroborated with our UHF-RF-acoustic imaging results (Figure 5b).

Immediately following in vivo imaging, we sacrificed the mice and excised the key organs (heart, liver, kidney, spleen, pancreas) and tumors to quantitatively assess the bio-distribution of the nanodroplets (Figure 5e,f). Figure 5f shows that the fluorescence signals in the tumors of group 1 was significantly higher than the rest of the groups (1.6 ± 0.4 -fold, 2.4 ± 0.6 -fold and 2.2 ± 0.6 -fold higher than groups 2, 3, and 4 respectively). In addition, fluorescence signals in the tumors of groups 3 and 4 were relatively low but detectable. This small amount of fluorescence signals could be due to non-specific uptake and/or the enhanced permeability and retention effects of the non-targeted nanodroplets⁵⁰. The slightly higher fluorescence signals in the tumors from group 2 with targeted nanodroplets compared to that from non-targeted nanodroplets in groups 3 and 4 (1.5 ± 0.4 -fold and 1.4 ± 0.4 -fold, respectively) is likely due to minimal GRPR expression of the DU145 cells, as observed in our in vitro evaluation (Figure 4b). Further, fluorescence quantification shows that in addition to tumor uptake, both targeted and non-targeted nanodroplets accumulated in the spleen and liver while negligible signals were detected in the other organs due

to the elimination by the reticuloendothelial system (Figure 5f). Pilot toxicity studies on healthy male mice show no adverse effect on the total blood count, relative to clinical pathology reference ranges (Supplementary Table S1), or visible damage to any tissue of key organs by histology (Figure 5g) after 14 days of intravenous nanodroplet injection (1×10^{10} nanodroplets).

Conclusions

UHF-RF-acoustic imaging is a low-cost, portable, and non-ionizing imaging technique for deep-tissue imaging. It provides imaging penetration near 10 centimeters with ultrasound resolution in tissue. The main obstacle to extend UHF-RF-acoustic imaging to molecular imaging application is the lack of imaging contrast agents. In this study, we developed an approach to produce the stable all liquid UHF-RF-acoustic contrast agent using saline and non-toxic surfactants. We show that our contrast agents can produce 1600 times higher UHF-RF-acoustic signals than concentration-matched iron-oxide nanoparticles. Finally, we demonstrated in vivo UHF-RF-acoustic molecular imaging using the targeted nanodroplets in a prostate cancer xenograft mouse model expressing GRPR. We expect these findings will accelerate the development of UHF-RF-acoustic molecular imaging for other cell surface targets and various disease states. In addition, the finding that perfluorocarbon liquid can stabilize high ionic liquid into stable nanodroplets will also benefit the development of nanocarriers to deliver high ionic prodrugs for disease treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1|. Experimental concept.

a, The spectral range of electromagnetic waves in the radio frequency range, where ultra-high frequency (UHF) ranges from 300 MHz to 1 GHz. **b**, Perspective view of the experimental setup for UHF-radio frequency acoustic (UHF-RF-acoustic) imaging of mice. The acoustic transducer was mounted on the side to image the x-y plane. The mouse was mounted vertically along the z axis. A translational stage can move the mouse in the $\pm z$ direction and the rotational stage rotates the mouse in the x-y plane. Two RF antennae emit RF pulses (160 ns) to excite the UHF-RF-acoustic signals. The same setup was also used for scanning tissue phantoms. **c**, The side view (x-z plane) of the experimental setup. **d**, The top view (x-y plane) of the experimental setup. **e**, Point spread function of the UHF-RF-acoustic system, the enlarged view shows the UHF-RF-acoustic signal pattern generated with a point source. **f**, One-dimensional point spread function of the UHF-RF-acoustic system. The inset shows the full-width-at-half-maximum (FWHM) is 1.2 mm.

Figure 2|. Preparation of UHF-RF-acoustic contrast agents with engineered saline nanodroplets. **a**, Experimental setup of the tube phantom for in vitro imaging with each tube containing different concentrations of electrolytes. **b**, UHF-RF-acoustic images of various types of electrolytes, the tubes contain 10 wt% concentration of NaCl, NaI, KCl, MgCl₂, and CaCl₂, and 4 wt% concentration of NaOH, with a diameter of 1 mm. **c**, UHF-RF-acoustic signal amplitude as a function of conductivity of the abovementioned electrolytes. The generated UHF-RF-acoustic peak amplitude follows a linear trend with the conductivity (grey dotted lines, $R^2=0.92$). Data are presented as mean values $\pm$ SD (N=5). **d**, A schematic illustration of nanodroplet synthesis using the double emulsion approach. **e**, Cryo-electron microscopy images show nanodroplets with NaCl_(aq) (25 wt%) core and perfluorocarbon shell (left) and a control droplet with only perfluorocarbon (right). **f**, Measured size distribution of the nanodroplets using dynamic light scattering, showing an average diameter of the nanodroplets $\sim$ 250 nm. **g**, Stability test of nanodroplets with a median diameter of 250 nm with various shells, including Δ : soybean oil (control), $\square$: perfluoropentane, $\circ$: perfluorohexane, $\diamond$: perfluoro-15-crown-5-ether, and ∇ : perfluorodecalin. Data are presented as mean values $\pm$ SD (N=25). By day 6, the soybean oil nanodroplets increase size by $\sim$ 215% relative to day 0 ($p < 0.0001$). By day 14, the diameters of

nanodroplets made of perfluorodecalin, perfluoro-15-crown-5-ether, perfluorohexane, and perfluoropentane nanodroplets increased by $18 \pm 15\%$ ($p = 0.2273$), $28 \pm 17\%$ ($p = 0.0751$), $65 \pm 17\%$ ($p < 0.0001$), and $103 \pm 18\%$ ($p < 0.0001$). **h**, Stability test of nanodroplets with perfluoropentane shell with different average diameters (□: 250 nm, ■: 450 nm, and ■: 800 nm). By day 14, 250 nm to 800 nm nanodroplets increases by $103 \pm 18\%$ ($p < 0.0001$), $139 \pm 18\%$ ($p < 0.0001$), $142 \pm 20\%$ ($p < 0.0001$). Data are presented as mean values $\pm$ SD (N=25).

Figure 3|. In vitro contrast-enhanced UHF-RF-acoustic imaging.

a, UHF-RF-acoustic signal amplitude of saline nanodroplets (open red circles) versus the initial concentration of $\text{NaCl}_{(\text{aq})}$, and the corresponding conductivity (filled blue squares, conductivity data adapted from Ref 27). Data are presented as mean values $\pm$ SD ($N=5$). **b**, UHF-RF-acoustic signal amplitude versus nanodroplet concentration with encapsulated 25 wt% saline, showing a linear correlation ($R^2=0.995$). Data are presented as mean values $\pm$ SD ($N=5$). The grey dashed line shows the UHF-RF-acoustic signal level of physiological saline (0.9 wt%). **c**, UHF-RF-acoustic signal amplitude versus aging time over 14 days. Nanodroplets (1.8×10^9 nanodroplets/mL) containing 25 wt% saline were measured, data are presented as mean values $\pm$ SD ($N=5$). **d**, UHF-RF-acoustic signal amplitude of tube phantoms with a 1-mm diameter. Six tubes were imaged; four tubes contain nanodroplets (1×10^9 nanodroplets/mL); one contains 20-nm gold nanoparticles (AuNPs, 5×10^{11} nanoparticles/mL); and one contains 100-nm iron oxide nanoparticles (Fe_3O_4 , 5×10^{11} nanoparticles/mL). Both AuNPs and Fe_3O_4 were prepared at 500 $\times$ higher concentration than the nanodroplets to bring their UHF-RF-acoustic signals above the noise level. The 1 $\times$ nanodroplets produce 3.2 ± 0.7 times higher UHF-RF-acoustic signals than the 500 $\times$ Fe_3O_4 nanoparticles (*, $p=0.0002$, $N=5$), and 5.1 ± 0.6 times higher signals than the 500 $\times$ gold nanoparticles (**, $p=1.4 \times 10^{-7}$, $N=5$). Data are presented as mean values $\pm$ SD. **e**, UHF-RF-acoustic signal amplitude of nanodroplets as a function of imaging depth in bovine tissue (photograph with setup on the left). Six 3-mm-diameter inclusions

were imaged, located at 0.5 cm, 1.8 cm, 2 cm, 2.8 cm, 3.5 cm, and 5 cm from the tissue surface. Each inclusion contains 1:1 volume ratio of nanodroplets (3×10^9 nanoparticles/mL with 25 wt% saline) and 12% gelatin. Data are presented as mean values $\pm$ SD (N=5). **f**, UHF-RF-acoustic imaging of a Stanford logo phantom. The phantom contains nanodroplets (8×10^9 nanodroplets/mL); the inclusion rotates for tomographic imaging.

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Figure 4|. GRPR-targeted nanodroplets showed specific targeting to prostate cancer cells and low toxicity in cell culture.

a, Extinction spectrum of the nanodroplets shows the optical absorption peak from indocyanine green (ICG) dye. **b**, Western blot analysis for GRPR expression in different prostate cancer cell lines. **c**, Cell viability test using non-targeted nanodroplets, showing no obvious reduction in cell viability when cells were incubated with up to 1×10^{11} nanodroplets/mL ($p=0.958$ relative to control), and low cell toxicity up to 3×10^{11} nanodroplets/mL (*, $p=0.009$, $N=30$) at 24 hours post incubation relative to cells incubated in the absence of nanodroplets. Data are presented as mean values $\pm$ standard deviation ($N=30$). **d**, Optical fluorescence imaging of ICG-labeled targeted (column 1) and non-targeted (column 2) nanodroplets incubated with GRPR⁺ (PC3) and GRPR⁻ (DU145) cells at 12 hours post incubation. Column 3 shows the optical fluorescence imaging of ICG-labeled targeted nanodroplets incubated with antibody-pre-blocked PC3 and DU145 cells, also at 12 hours post incubation. Both PC3 and DU145 cells express green fluorescent

protein (GFP). ICG dyes on nanodroplets and GFP of the cells were shown as red and green, respectively.

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Figure 5]. In vivo UHF-RF-acoustic molecular imaging using targeted nanodroplets.

a, Configuration of the tomographic mouse imaging. **b**, In vivo UHF-RF-acoustic imaging with subcutaneous GRPR⁺ tumors (PC3) using targeted nanodroplets (group 1, N=5), GRPR⁻ tumors (DU145) using targeted nanodroplets (group 2, N=5), PC3 tumors using non-targeted nanodroplets (group 3, N=5), and DU145 tumors using non-targeted nanodroplets (group 4, N=5). **c**, Comparison of UHF-RF-acoustic signal amplitude in the tumor region with targeted and non-targeted nanodroplets, showing the strongest signals from the targeted nanodroplets with PC3 tumors (*, $p=0.007$; **, $p=0.404$). Data are presented as mean values $\pm$ SD (N=5). **d**, Epifluorescence imaging of mice in groups 1 to 4 at 48 hours post injection, showing the strongest ICG fluorescence signals from PC3 tumor bearing mouse with the GRPR-targeted nanodroplets. The scanning area is 66 mm $\times$ 50 mm. **e**, Fluorescence imaging (top) of harvested main organs and tumor from one mouse in group 1, showing that the nanodroplets mainly accumulate at the tumor and the spleen. Fluorescence imaging (bottom) of the harvested tumors from one mouse from each group, showing the strongest signal from the mouse in group 1, demonstrating their highest targeting specificity and efficiency. **f**, ICG fluorescence signals from the major organs and tumors from the 4 groups of mice, indicating that the nanodroplets mainly accumulate at the

tumor and the spleen, but only GRPR-targeted nanodroplets show highest binding specificity at the tumor site (*, $p=0.006$; **, $p=0.0005$; ***, $p=0.0007$; ****, $p=0.017$; and *****, $p=0.051$). Data are presented as mean values $\pm$ SD (N=5). **g**, Immunohistochemical tissue sections of liver, kidney, and spleen from mice with non-targeted nanodroplet or saline injections (100 μ L, 1×10^{11} nanodroplets/mL) for 2 weeks, stained with hematoxylin and eosin (H&E) and Perls' Prussian Blue. There was no noticeable tissue damage from the intravenous nanodroplet injections.

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