Detection of pancreatic tumors *in vivo* with tumor targeted mesoporous silica-coated gold nanorods by multispectral optoacoustic tomography

Anil Khanal¹, Christopher Ullum¹, Charles W Kimbrough², Nichola C. Garbett¹, Joseph A. Burlison¹, Molly W. McNally¹, Phillip Chuong¹, Ayman S. El-Baz³, Jacek B. Jasinski³, and Lacey R. McNally¹ (✉)

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Mesoporous silica-coated gold nanorods targeted with Syndecan-1 selectively target IGF-1R positive pancreatic cancer cells as detected using multispectral optoacoustic tomography.
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ABSTRACT

Multispectral optoacoustic tomography is an emerging imaging technology that offers several advantages over traditional modalities, particularly in its ability to resolve optical contrast at depth on a microscopic scale. While potential applications include the early detection of tumors below clinical thresholds set by current technology, the lack of tumor-specific contrast agents limits the use of MSOT imaging. Therefore, we constructed highly stable nano-contrast agents by coating gold nanorods (GNRs) with either polyacrylic acid (PAA) or amine-functionalized mesoporous silica (MS). Syndecan-1 targeting ligand, which has been shown to target up-regulated insulin-like growth factor 1 receptor (IGF1-R) in pancreatic tumors, was conjugated on the surface of PAA coated GNRs (PAA-GNRs) or MS coated GNRs (MS-GNRs) to create tumor targeted nanoparticles. The optoacoustic (OA) signal enhancing effect of the tumor targeted, coated GNRs was then examined using MSOT, while tumor targeting in vitro was assessed with flow cytometry. In S2VP10L cells (positive for IGF1-R), the Syndecan-1 MS-GNRs (Syndecan-MS-GNRs) gave 10X higher OA signal than Syndecan-1 PAA-GNRs (Syndecan-PAA-GNRs). Minimal binding was observed in MiaPaca-2 cells (negative for IGF1-R). Syndecan-MS-GNRs were then tested in vivo using a murine orthotopic pancreatic cancer models, and in mice implanted with S2VP10L cells the Syndecan-MS-GNRs demonstrated significantly more accumulation in tumors compared to off-target organs such as the liver. Mice implanted with the IGF1-R negative MiaPaca-2 cells did not demonstrate specific tumor targeting. In summary, we report that targeted nano-contrast agents (Syndecan-MS-GNRs) can provide a high-resolution signal amplifier that minimizes off-target effects and successfully detects orthotopic pancreatic tumors in vivo using MSOT.
1. Introduction

Despite intensive research efforts, the last four decades has seen relatively modest advancements in the early detection and treatment of pancreatic cancer, and the overall mortality from this disease has essentially remained unchanged. The aggressive underlying biology of pancreatic tumors makes this a very difficult disease to treat. Upwards of 80% of patients will have metastases by the time that primary tumors are clinically detectable, as current imaging modalities fail to accurately identify lesions less than 2-3 cm in size. In addition, the desmoplastic stroma and poor vascularity of these tumors leads to limited tissue penetration of systemic chemotherapies and contrast agents. The combination of enhanced imaging via MSOT with tumor-specific nano-contrast agents could mitigate these impediments.

Optoacoustic (photoacoustic) imaging is an emerging technology that provides molecular information at clinically relevant depths with a higher resolution than many existing techniques. Multispectral optoacoustic tomographic imaging is unique in that it resolves optical contrast, but the resolution obeys the rules of ultrasonic diffraction. Essentially, as a result of the photoacoustic effect, excitation of tissue by pulsed laser light generates an acoustic signal that is detectable by MSOT. Thus, optoacoustic methods render photon scattering irrelevant to image formation, and can generate images with sub-mm resolution at a greater depth than optical imaging techniques. Ultimately, this enables the capability for novel high-resolution insights into the biological function of entire tumors, organs, and systems.

Gold nanorods (GNRs) are mostly employed as exogenous contrast agents in optoacoustic (OA) imaging to improve image contrast and depth penetration, since GNRs display strong plasmon resonance peaks in the near infra-red that facilitates tissue penetration of radiation up to a few centimeters. GNRs have a relatively high near infra-red absorption coefficient as compared to carbon nanotubes, and are highly stable in comparison to silver nano-prisms. In addition, GNRs have a more advantageous size scale for in vivo molecular imaging and a sharper absorption peak than other gold nanoparticles (gold nano-shells and gold nano-prisms), which make GNRs promising contrast agents in OA imaging. However, GNRs change shape and become more spherical as they absorb OA laser fluence during in vivo imaging, which significantly decreases the contrast and resolution of images. Encapsulating GNRs in amine-functionalized mesoporous silica (MS) enhances the thermal stability, and protects GNRs from thermal deformation and reshaping. Encapsulation with polyacrylic acid (PAA) enhances thermal conductivity of the surface layer in PAA coated GNRs. Although GNRs have potential application as targeted contrast agents, their signal must be amplified using a combination of coatings to facilitate detection of tumors.

Current utilization of multispectral optoacoustic tomography (MSOT) is hindered by a lack of tumor-specific contrast agents and limited testing tumors at sub-surface depths. In this study, we had two aims: 1) compare two strategies (PAA or MS) of coating GNR to result in higher optoacoustic signal, and 2) determine if conjugation of Syndecan-1 ligand to PAA-GNR or MS-GNR will improve tumor targeting of orthotopic pancreatic cancer in vivo as detected using multispectral optoacoustic tomography. With this in mind, we constructed nano-contrast agents composed of polyacrylic acid (PAA) or mesoporous (MS) shell coated GNRs. Additionally, Syndecan-1 ligand was conjugated to the surface of PAA coated GNRs (Syndecan-PAA-GNRs) and MS coated GNRs (Syndecan-MS-GNRs) in order to specifically target pancreatic tumor cells that overexpressed insulin-like growth factor 1 receptor (IGF1-R). Although Syndecan-1 ligand binds to IGF1-R, it has not been used as a targeting moiety for nanoparticles. Characterization of the targeted GNRs, as well as both in vitro and in vivo binding experiments, were performed to evaluate the optoacoustic signal enhancement and tumor targeting capability of these contrast agents. To our knowledge, this is the first successful use of targeted
nano-contrast agents to facilitate detection of pancreatic cancer in vivo using MSOT.

2. Materials and methods

2.1. Materials

Cetyltrimethylammonium bromide (CTAB) (≥99% for molecular biology), gold (III) chloride trihydrate (HAuCl₄·3H₂O), sodium borohydride (NaBH₄), silver nitrate (AgNO₃), L-Ascorbic Acid (L-AA), poly-acrylic acid (PAA, MW~ 450,000 g mol⁻¹), N-Hydroxysuccinimide (NHS), N-(3-Dimethyaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), tetraethyl orthosilicate (TEOS), and (3-aminopropyl) triethoxysilane (APTES), methanol(99.8 %), ethanol(99.5 %), Osmium tetroxide solution (4% in H₂O) and glutaraldehyde solution (25% in H₂O) were purchased from (Sigma-Aldrich, St. Louis, MO) and used as received. Dulbecco’s Modified Eagle’s Medium (DMEM), L-glutamine and RPMI medium were purchased from (Life Technologies, Grand Island, NY). Fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and Syndecan-1 ligand (ProSpec Protein Specialists, Rehovat, Israel) were used as received.

2.2. Synthesis of CTAB coated GNRs (CTAB-GNRs)

Gold nanorods (GNRs) were synthesized via the seed-mediated method in aqueous solution [27]. To prepare the GNRs seeds, freshly prepared-ice cold NaBH₄ (0.3 mL, 10 mM) was added to a mixture of CTAB (2.5 mL, 0.2 M) and HAuCl₄ (2.5 mL, 0.6 mM) under vigorous stirring. The solution turned from yellow to brown indicating formation of gold nanoparticle seeds with stirring continued for 10 minutes. The seed solution was then placed into a water bath kept at 27 °C. For the GNR growth solution, CTAB (0.2 M, 100 mL) was mixed with HAuCl₄ (1.5 mL, 50 mM) and AgNO₃ (0.2 mL, 50 mM). A mild reducing agent, ascorbic acid (1.25 mL, 0.8 mM), was then added, and the color of the solution rapidly changed from bright yellow to colorless. To initiate GNR growth, 200 μL of gold seed solution was added into the GNR growth solution. The reaction mixture was swirled, and then allowed to sit overnight in a 27 °C water bath. Excess CTAB was removed by centrifugation (10000 g, 30 min, and 3 times) and finally, CTAB coated GNRs (CTAB-GNRs) were dispersed in 25 mL of ultrapure water Milli-Q filtered, (Millipore, Billerica, MA).

2.3. Synthesis of MS-GNRs

Before silica coating, the pH of CTAB-GNRs (10 mL) was adjusted to pH 9 by adding 0.10 mL of 0.10 M NaOH with continuous stirring. This was followed by the addition of three aliquots of 30μL of 20% TEOS in methanol and 10μL of 2% APTS in methanol at 30 min intervals [27]. The reaction was allowed to proceed for 24 h. The resulting amine-functionalized mesoporous silica-coated gold nanorods (MS-GNRs) were then washed with methanol (99%) and ethanol (99%) five times, and the nanoparticles dispersed in 1.5 mL of Milli-Q filtered water.

2.4. Synthesis of PAA-GNRs

PAA coating on GNRs was achieved using the layer-by-layer (LBL) approach in phosphate buffer (10 mM, pH 7.4) [28]. In brief, PAA solution (10 g/L) was prepared in a 1 mM NaCl solution and sonicated for 30 min. The CTAB-GNRs (6.5 mg/mL) were then added drop-wise to the PAA solution (v/v = 1:3) under vigorous stirring for 6 h to get PAA-GNRs. The PAA-GNRs were separated by centrifugation (10000 g, 30 min, and 3 times) to remove excess PAA and then re-dispersed in 2 mL of phosphate buffer (10 mM, pH 7.4).

2.5. Conjugation of Syndecan-1 ligand to PAA-GNRs or MS-GNRs

Syndecan-1 ligand was conjugated onto the PAA-GNRs or MS-GNRs by EDC-NHS chemistry in phosphate buffer (10 mM, pH 7.4) [29]. Briefly, the amine group of Syndecan-1 ligand (0.74 mM, 2.7 μL) was conjugated to PAA carboxylic groups of PAA-GNRs by adding NHS (1 mM, 20 μL) and EDC (1 mM, 20 μL) to 5 mL of PAA-GNRs (6.5 mg/mL) in phosphate buffer (10 mM, pH 7.4), and the mixture was stirred gently overnight (Scheme 1). Syndecan-1 ligand conjugated PAA-GNRs (Syndecan-PAA-GNRs) were separated by centrifugation (2129.79 g, 30 min, and 2 times) and re-dispersed in phosphate buffer (10 mM, pH 7.4).
Scheme 1: Coating of GNRs. (A) Syndecan-PAA-GNRs were synthesized by coating GNRs with PAA and further conjugating surface of PAA with Syndecan-1 ligand. (B) Syndecan-MS-GNRs were synthesized by coating GNRs with MS by using TEOS and APTES. Syndecan-1 ligand was conjugated on the surface of MS-GNRs.

Likewise, the Syndecan-1 ligand was conjugated to mesoporous silica-coated gold nanorods (MS-GNRs) in phosphate buffer (10 mM, pH 7.4). The Syndecan-1 ligand (0.74 mM, 2.7 μL) was activated by adding EDC (1mM, 20 μL) and NHS (1mM, 20 μL) at pH 7.4, followed by stirring overnight. The resulting solution was mixed with 5 mL of MS-GNRs (6.5 mg/mL, at pH 7.4), and stirred overnight. The Syndecan-1 ligand conjugated MS-GNRs (Syndecan-MS-GNRs) were separated by centrifugation (2129.79 g, 30 min, and 2 times), then re-dispersed in phosphate buffer (10 mM, pH 7.4). Both Syndecan-MS-GNRs and Syndecan-PAA-GNRs were diluted further to get the desired concentration for experiments described here.

2.6. Stability of PAA-GNRs and MS-GNRs

The stability of all synthesized GNRs in DMEM media was assessed by UV-Vis spectroscopy (Cary 100, Varian, CA). For this, 1 mL of all of synthesized GNRs was mixed with 2 mL of DMEM media. Stability was assessed by comparing absorbance scans obtained in the range 400-900 nm after 1, 18, and 48 h of mixing.

2.7. Zeta-potential measurements

Electrophoretic mobility (μE) of CTAB-GNRs, PAA-GNRs and MS-GNRs were measured at 25 °C with Zetasizer Nano-ZS (Malvern Instruments, Ltd., Malvern, UK). The zeta-potential of the samples was calculated from $\mu E$ using following Smoluchowski’s equation:

$$\mu E = \frac{\zeta \epsilon}{\eta}$$

where $\zeta$ is the zeta-potential, $\epsilon$ the permittivity of solvent, and $\eta$ the viscosity of solvent.

2.8. Cell culture, Western blot, and flow cytometry

2.8.1. Cell culture

The pancreatic adenocarcinoma cell lines S2VP10L (positive for IGF1-R) and MiaPaca-2 (negative for IGF1-R) were cultured with DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Atlanta Biological, Atlanta, GA) and 1% L-glutamine, and the cells were grown at 37° C in a humidified incubator with 5% CO$_2$ [29]. Luciferase expression in pancreatic cancer cells was confirmed in our previous work [30].

2.8.2. Western Blot for IGF1-R

Cells were lysed in a buffer solution containing 1% NP-40, 1% phosphatase inhibitor and 1% protease inhibitor in nuclease free-water. Lysates were centrifuged at 13,300 x g for 10 min at 4° C. The total protein in the lysates was quantified using the Bradford assay [29]. Approximately 50 μg of protein from S2VP10L (positive for IGF1-R) and MiaPaca-2 (negative for IGF1-R) samples were dissolved in loading buffer. Proteins were separated by NuPage 4–12% Bis-Tris gel, and then transferred onto nitrocellulose membranes using iBlot (Life Technologies, Grand Island, NY). The membranes were blocked with Li-Cor blocking buffer (Li-Cor, Lincoln, NE). Proteins were incubated with IGF1-R (Abcam, Cambridge, UK) and B-actin at dilutions of 1:1000 and 1:3000, respectively. The membranes were incubated overnight at 4° C, then washed...
three times using Tris-buffered saline with Tween 20 (TBS) and incubated with secondary antibodies, anti-rabbit IgG or anti-mouse (Li-Cor) diluted 1:2500 at room temperature. Membranes were scanned and analyzed using Li-Cor Odyssey.

2.8.3. Flow cytometry

Indocyanine green (ICG) was embedded into Syndecan-MS-GNRs or MS-GNRs as previously described [20]. Typically, 20 μL of ICG (10 mM) in ethanol was added to 5 mL of Syndecan-MS-GNRs or MS-GNRs (6.5 mg/mL) in phosphate buffer (10 mM, pH 7.4) and stirred overnight. The nanoparticles were collected by centrifugation (2129.79 g, 30 min, and 4 times) and re-dispersed in phosphate buffer (10 mM, pH 7.4).

The pancreatic cancer cell lines S2VP10L (positive for IGF1-R) and MiaPaca-2 (negative for IGF1-R) were seeded at a density of 4×10^5 cells/well in 6-well plates. Cells were treated with 20 μL of 100 nM Syndecan-MS-GNRs (containing 0.01 mM indocyanine green) or MS-GNRs (containing 0.01 mM indocyanine green) for 1 h. The antibody-blocked IGF1-R wells were treated with 10 μL anti-IGF1-R antibody (Abcam, Burlingame, CA) for 1 h prior to addition of nanoparticles. Then, cells were scraped and washed with PBS for preparation for flow cytometric analysis of nanoparticle uptake using the BD FACSCanto (BD Biosciences, San Jose, CA). The APC-Cy7 filter setting detected the cells positive for indocyanine green. Raw data was analyzed using FlowJo software (FlowJo, Ashland, OR). The Wilcoxon sign-rank test was conducted to evaluate significance (p<0.05).

2.9. Optoacoustic tissue phantoms

The tissue phantoms were used to observe the cellular uptake and signal amplification of nanoparticles using MSOT. Nanoparticles at concentration 1.1μg/μL (Syndecan-MS-GNRs, MS-GNRs, Syndecan-PAA-GNRs, and PAA-GNRs) were incubated with S2VP10L (positive for IGF1-R) or MiaPaca-2 (negative for IGF1-R) cells for 2 h at 37°C and 5% CO₂. Cells were then washed five times with phosphate buffer (10 mM, pH 7.4) and cellular uptake by the tissue phantoms determined by MSOT. The tissue phantoms were constructed by using the following procedures: Fixed cylindrical phantoms of 2 cm diameter were prepared using a gel made from distilled water containing Agar (Sigma Aldrich, St. Louis, MO) for jellification (1.3% w/w) and an intralipid 20% emulsion (Sigma Aldrich, St. Louis, MO) for light diffusion (6% v/v), resulting in a gel presenting a reduced scattering coefficient of μ's ≈ 10 cm⁻¹. A cylindrical inclusion containing the Syndecan-MS-GNRs approximately 3 mm diameter was put in the middle of the phantoms.

Imaging of the phantoms was done at a single position located approximately in the middle of the phantoms. Data acquisition was performed at wavelengths of 680, 710, 730, 740, 760, 770, 780, 800, 850, 900 nm, using 10 averages per wavelength resulting in 1 s acquisition time per wavelength. Nanoparticle uptake was measured from a region of interest method using MSOT images. Data obtained in MSOT arbitrary units (a.u.) was statistically compared using ANOVA.

2.9. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) Measurements

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR Perkin-Elmer, Spectrum series 100 spectroscopy, Norwalk, CT) was used to characterize MS-GNRs and to confirm the conjugation of Syndecan-1 ligand to MS-GNRs. For this, 5 mL of Syndecan-MS-GNRs or untargeted MS-GNRs solution in phosphate buffer (10 mM, pH 7.4) was centrifuged (2129.79 g, 30 min), then dried using a Savant RVT 100
Refrigerated Vapor Trap (Holbrook, NY) for 3h. 10 mg of Syndecan-MS-GNRs or untargeted MS-GNRs was mixed with potassium bromide FT-IR grade, (Sigma-Aldrich, St. Louis, MO) and pressed with a pressure of 80 psi. Each FTIR spectrum was collected after 60 scans at a resolution of 2 cm\(^{-1}\) from 400 to 4000 cm\(^{-1}\).

2.9.1. Evaluation of coated GNRs alone or within cells using transmission electron microscopy

The initial morphology of PAA-GNRs or MS-GNRs was observed using a transmission electron microscope (TEM; Tecnai-F20), (FEI Co., Eindhoven, Netherlands). For this, 50 μL of PAA-GNRs or MS-GNRs were dropped onto copper-mesh grids (Electron Microscopy Science, Hatfield, PA) and dried at room temperature for 24h prior to imaging.

Cellular uptake of Syndecan-MS-GNRs in S2VP10L (positive for IGF1-R) cells was observed by transmission electron microscopy (TEM). First, S2VP10L (positive for IGF1-R) cells were grown at 37 °C and 5% CO\(_2\) in DMEM with 10% fetal bovine serum and 1% L-glutamine \(^{[16]}\). Then, 1.1μg/μL of Syndecan-MS-GNRs was incubated with S2VP10L (positive for IGF1-R) cells for 2 h at 37 °C and 5% CO\(_2\). Afterwards, cells were washed five times with a phosphate buffer (10 mM, pH 7.4) and fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C for 24 hours. Cells were post-fixed in 1% osmium tetroxide for 1 h at room temperature. The samples were then washed in distilled water and dehydrated by a graded ethanol series. Samples were infiltrated using propylene oxide and EPO epoxy resin Embed 812, (Electron Microscopy Sciences, Hatfield, PA), and finally embedded with epoxy resin. The samples were then loaded into BEEM capsules (Electron Microscopy Sciences, Hatfield, PA) and polymerized at 60°C for 24 h. Thin-sections were cut using a diamond knife on a Leica-Reichert Ultracut E ultra-microtome and collected on 200 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). Sections were stained with uranyl acetate and lead citrate (Electron Microscopy Sciences, Hatfield, PA). Images were collected using a Phillips CM-10, transmission electron microscope at 80kv, equipped with a 15 megabyte SIA digital camera.

2.9.2. Orthotopic implantation of pancreatic cancer cells within SCID mice

Female severe combined immunodeficient mice 4 weeks of age were used for this study in strict adherence to a University of Louisville Institutional Animal Care and Use Committee (IACUC) approved protocol. Mice were placed on 2920X alfalfa free-rodent diet (Harlan Laboratories, Indianapolis, IN) to reduce background signal during imaging. An established model for orthotopic cell implantation into the mouse pancreas was followed as previously described \(^{[26, 30]}\). Briefly, 10 mice were anesthetized with isoflurane and hair was removed from the abdomen using Nair with Aloe (Church & Dwight Co, Princeton, NJ), followed by washing with warm water. The abdomen was then prepped with betadine.

A 1-cm incision was made in the left upper quadrant, with the pancreas exposed by retraction of the spleen. A solution of 1.5 x 10\(^5\) cells/30μL of S2VP10L (positive for IGF1-R) or (2.5 x 10\(^6\) cells/30μL) of MiaPaca-2 (negative for IGF1-R) cells in in serum-free RPMI medium was drawn up using a 28-gauge needle and injected into the tail of the pancreas. A sterile cotton tipped applicator was held over the injection site for 30 seconds to prevent peritoneal leakage. The organs were returned to the abdomen with the skin and peritoneum closed in a single layer using 5-0 nylon sutures. Mice recovered underneath a warming blanket and were returned to their cages with food and water ad
After regaining full mobility.

2.9.3. Tumor monitoring with bioluminescence imaging

Bioluminescence imaging was used immediately following surgery to assess potential leakage of cells from orthotopic implantation with the AMI-1000-X instrument (Spectral Imaging Instruments, Tucson, AZ). Mice received ip injection of 2.5 mg luciferin 10 min prior to imaging. Mice with signs of peritoneal leakage were excluded from further study as in [13, 26, 30]. Sutures were removed after 5 days to prevent artifacts for subsequent imaging studies. Tumor size was assessed again at 7 days post-op (S2VP10L, positive for IGF1-R) or 18 days post-op (MiaPaca-2, negative for IGF1-R) and prior to injection of Syndecan-MS-GNRs particles. Ultimately, 5 mice (S2VP10L, positive for IGF1-R) and 3 mice (MiaPaca-2, negative for IGF1-R) were selected for in vivo MSOT imaging based on mean bioluminescent signal from pancreatic tumors.

2.9.4. Evaluation of Syndecan-MS-GNRs accumulation in vivo assessed using multispectral optoacoustic tomography

The inVision-256TF multispectral optoacoustic tomography system (iThera Medical, Munich, Germany) was used for real-time detection of Syndecan-MS-GNRs biodistribution within orthotopic pancreatic xenografts. Mice were anesthetized with 1.6% isoflurane inhalant delivered in 0.8 L medical air and 0.1 L O2, then depilated using a combination of shaving and application of Nair, which was removed with moist gauze [13]. Anesthetic depth was maintained throughout the image acquisitions with mice oriented ventral side up in the animal holder. Whole-body imaging capture was performed 4h after injection of Syndecan-MS-GNRs (1.1 μg/μL in 10 mM phosphate buffer, pH 7.4 with 0.5 % NaCl ) using transversal slices with a 0.2-mm step from the liver to the kidney (38-56 mm), at wavelengths of 680, 710, 730, 740, 760, 770, 780, 800, 850, 900 nm for each position, using 25 averages per wavelength with an acquisition time of 10 μsec per frame in order to minimize the influence of animal movement in the images. Excitation of the Syndecan-MS-GNRs was conducted using a tunable parametric oscillator pumped by an Nd: YAG laser. The pancreatic tumor was identified by a live-feed screen preview multispectral signal (MSP).

2.9.5. MSOT image reconstruction and evaluation of nanoparticle uptake and biodistribution

Images were reconstructed using the multispectral processing, along with ViewMSOT 3.5 software. Data were optimized using high-resolution (75 μm) back projection reconstruction, which was done prior to applying the multispectral processing using linear regression. Orthogonal views were also created using ViewMSOT 3.5. Region of interest analysis was utilized to determine nanoparticle uptake and biodistribution within the pancreas tumor, kidney, and liver [16]. Peak intensity was determined in each of the three organs and statistically compared using ANOVA with significance at p<0.05.

3. Results and Discussion

3.1. Preparation and characterization of PAA-GNRs or MS-GNRs

Exogenous agents having large optical absorption are frequently introduced as optoacoustic (OA) contrast agents to improve OA signal and to enhance image quality compared to endogenous contrasts such as hemoglobin [13, 31]. Metal nanoparticles are frequently employed because of their tunable optical absorption properties at near infra-red region and their resistance to photobleaching, a problem commonly associated
with dyes [32].

In optoacoustic (OA) imaging, an OA wave is generated by the thermal expansion of contrast agents after absorption of a short laser pulse. Because optical absorption is one of the critical criteria for the generation of an OA signal, it is essential to maintain both the size and shape of GNRs [19, 33]. The laser pulses and thermal expansion of GNRs can reshape and deform the uncoated GNRs, resulting in poor penetration depth and alteration of the contrast agents [33]. This deformation of GNRs also increases their toxicity profile [19]. To improve the potential of GNRs as contrast agents for optoacoustic imaging, we compared two coating strategies, PAA and MS. These two methods were selected because the literature suggests Polyacrylic acid (PAA) increases the thermal conductivity of the surface layer in PAA coated GNRs [21] while coating of GNRs with MS enhances the stability of GNRs under high-fluence laser pulses [19].

GNRs have high absorption in the tissue optical window (650–1100 nm), which helps maximize the depth of OA imaging. Coatings of PAA or MS onto GNRs maintain this optical window as confirmed by UV-vis absorbance spectroscopy (Figure 2A). The surface modification of GNRs by PAA and MS shifts the longitudinal surface plasmon resonance of GNRs from 723 nm for uncoated GNRs to 741 nm and to 755 nm, respectively. The red shifts of the longitudinal surface plasmon resonance of GNRs results from changes in the surrounding environments of GNRs upon coating with MS or PAA.

**Figure 1:** Transmission electron micrograph (TEM) of GNRs. (A) PAA-GNRs with PAA shell thickness (1.5 ± 0.5 nm). (B) MS-GNRs with MS shell (3.25 ± 0.254 nm). The average length and width of GNRs were PAA-GNR (24 ± 3.15 nm and 10.0 ± 0.33 nm) and MS-GNR (27.1 ± 2.4 nm and 12.8 ± 1.5 nm).

Successful coating of PAA or MS onto GNRs was observed by transmission electron microscopy (TEM) (Figure 1). The shell thickness of PAA in PAA-GNRs was 1.5 ± 0.5 nm, while MS thickness in MS-GNRs was 3.25 ± 0.25 nm. The average length and width of GNRs were PAA-GNR (24 ± 3.15 nm and 10.0 ± 0.33 nm) and MS-GNR (27.1 ± 2.4 nm and 12.8 ± 1.5 nm).

**Fig. 2:** Absorption spectra of GNRs. (A) CTAB-GNRs, PAA-GNRs and MS-GNRs have a longitudinal plasmon resonance band at 723, 741 and 755 nm, respectively. (B) Instability and aggregation of CTAB-GNRs are apparent after 1 hr. (C) Stability of PAA-GNRs is shown up to 2 days. (D) Stability of MS-GNRs is shown up to 2 days. For the stability of experiments, nanoparticles (1mg/mL) were mixed with DMEM solution.

In this study, the synthesized pure GNRs contained an unstable cetyltrimethylammonium bromide
(CTAB) bilayer on the surface of the GNRs, which induces aggregation of CTAB-GNRs and contributes to cytotoxicity to human cells due to the highly cytotoxic property of free CTAB [34]. By encapsulating the CTAB-GNRs in PAA or MS, the GNRs are more suitable for biomedical application, because both PAA and MS mask the CTAB bilayer and reduce potential toxicity [19, 28]. The CTAB-GNRs had a zeta potential of +10 mV due to the presence of positively charged CTAB, but encapsulation of GNRs in PAA and MS resulted in zeta potentials of -59 and -9 mV, respectively, indicating the successful coating of PAA or MS in GNRs.

3.2. Stability of PAA-GNRs and MS-GNRs

To design effective contrast agents, the first consideration should be stability of these contrast agents in vitro. In this context, the stability of contrast agent was examined in biological media. The uncoated GNRs were highly unstable in cell culture medium over time and deemed unsuitable for in vivo use (Figure 2B). Surface modification of GNRs by MS or PAA substantially enhanced the stability as indicated by the longitudinal surface plasmon resonance band which remained unchanged for PAA-GNRs and MS-GNRs, when treated with Dulbecco’s Modified Eagle’s Medium (DMEM) for 2 days (Figure 2C-D).

3.3. Western blot

Previously, Syndecan-1 ligand has been demonstrated to bind to insulin-like growth factor 1 receptor (IGF1-R) [23-25]. Examination of IGF1-R expression on S2VP10L and MiaPaca-2 cells using Western blot demonstrated that IGF1-R was significantly expressed in S2VP10L cells, while MiaPaca-2 cells lacked IGF1-R (Fig. S1 in the Electronic Supplementary Material (ESM). Thus, we employed both S2VP10L (positive for IGF1-R) and MiaPaca-2 (negative for IGF1-R) cells to evaluate receptor-specific binding of Syndecan-1 ligand targeted particles.

3.4. Optoacoustic tissue phantoms

Because two of the aims of our study were to determine which coating of GNRs would produce higher optoacoustic signal and if Syndecan-1 ligand improved cellular binding, we compared optoacoustic signaling of untargeted PAA-GNRs and MS-GNRs with Syndecan-PAA-GNRs and Syndecan-MS-GNRs in tissue phantoms where signal was detected using MSOT. Targeting cancer surface cell receptors using a specific binding ligand is one of the most successful strategies to detect and image tumor cells, since these strategies enhance contrast signal in optoacoustic imaging by effectively accumulating in the cancer cells [35]. Syndecan-1 ligand was conjugated to the surface of PAA-GNRs or MS-GNRs by N-Ethyl-N-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) (Scheme 1). To evaluate the in vitro targeting effect of both Syndecan-1 ligand conjugated PAA-GNRs (Syndecan-PAA-GNRs) and Syndecan-1 ligand conjugated MS-GNRs (Syndecan-MS-GNRs), nanoparticles with or without Syndecan-1 ligands (1.1 µg/µL) were incubated with S2VP10L (positive for IGF1-R) or MiaPaca-2 (negative for IGF1-R) cells in vitro for 2 h and washed five times to remove unbound nanoparticles. The cellular uptakes of these contrast agents were observed by multispectral optoacoustic tomography (MSOT) tissue phantom (Figure 3A).

The Syndecan-MS-GNRs effectively accumulated in S2VP10L cells (positive for IGF1-R), indicated by the increased signal (yellow) compared to Syndecan-PAA-GNRs, untargeted MS-GNRs, and untargeted PAA-GNRs (Figure 3A). Accumulation was not seen in the MiaPaca-2 cells. Furthermore, MSOT tissue phantoms demonstrated that the
Syndecan-MS-GNRs substantially higher OA signal, and were almost 10-fold higher than Syndecan-PAA-GNRs in S2VP10L cells (Figure 3B). Consequently, Syndecan-MS-GNRs are effective in serving as nano-amplifiers to enhance OA signal and an effective cancer-specific contrast agents in MSOT.

![Figure 3](image)

**Figure 3**: Visualization of GNRs in S2VP10L (positive for IGF1-R) and MiaPaca-2 (negative for IGF1-R) cells using phantoms with multispectral optoacoustic tomography (MSOT). (A) (Top) S2VP10L (positive for IGF1-R) cells and (Bottom) MiaPaca-2 (negative for IGF1-R) cells treated with MS-GNRs and Syndecan-MS-GNRs or PAA-GNRs and Syndecan-PAA-GNRs, respectively. (B) Intensity of experimental phantoms in MSOT units (a.u). Syndecan-MS-GNRs resulted in significantly higher signal (p<0.05).

**3.5. Flow cytometry**

Tissue phantoms suggest that Syndecan-MS-GNRs significantly enhanced OA signal by binding with IGF1-R in S2VP10L. To further confirm these results, competitive inhibition of Syndecan-MS-GNRs to IGF1-R was conducted using a blocking agent (anti-IGF1-R antibody) and further evaluated in MiaPaca-2 (negative for IGF1-R) cells, respectively. The addition of anti-IGF1-R antibody effectively blocks binding of Syndecan-MS-GNRs in S2VP10L (positive for IGF1-R) cells, but does not alter MS-GNRs non-specific binding (Fig. S2 in the Electronic Supplementary Material (ESM). Histograms of mean fluorescence intensity counts demonstrate significant Syndecan-MS-GNRs uptake in the S2VP10L (positive for IGF1-R) cells (503.4 counts), compared to 64.1 in MiaPaca-2 (negative for IGF1-R) cells. Upon addition of anti-IGF1-R blocking antibody, Syndecan-MS-GNRs binding to S2VP10L (positive for IGF1-R) cells was 323.7, resulting in a 35% reduction in signal. Wilcoxon sign-rank test was conducted to evaluate significance. Although this was not statistically significantly different (p>0.05), the reduction of Syndecan-MS-GNRs uptake after the addition of anti-IGF1-R indicates biological significance. Furthermore, the increase in uptake of Syndecan-MS-GNRs in comparison to MS-GNRs was significantly higher in S2VP10L (positive for IGF1-R) cells than MiaPaca-2 (negative for IGF1-R) (p<0.05).

**3.6. ATR-FTIR measurements for MS-GNRs and Syndecan-MS-GNRs**

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) measurements were performed to characterize MS-GNRs and to confirm the conjugation of Syndecan-1 ligand in MS-GNRs (Figure 4). The MS-GNRs show the ATR-FTIR bands due to amine functionalized mesoporous silica (MS). Amine functionalized mesoporous silica (MS) GNRs exhibited the vibration band at 3432 cm\(^{-1}\) due to the surface hydroxyl and amine groups of mesoporous silica.
The bands at 2924 cm\(^{-1}\) and 2873 cm\(^{-1}\) were assigned to stretching mode of \(\text{CH}_2\) due to grafting of amino propyl groups in the mesoporous silica. The Si–O–Si bands that originated from mesoporous silica (SiO\(_2\)) were observed at around 1102 cm\(^{-1}\) with a shoulder at 1250 cm\(^{-1}\) due to asymmetric Si–O–Si stretching modes. A band at 795 cm\(^{-1}\) assigned to symmetric Si-O-Si stretching modes and the peak at 470 cm\(^{-1}\) denoted the Si–O out-of-plane deformation band \([36]\). A band at 1625 cm\(^{-1}\) was observed in MS-GNRs due to the overlapping of OH and N-H groups. The Syndecan-MS-GNRs showed a band at 1694 cm\(^{-1}\) (C=O amide I band), which verified the presence of the Syndecan ligand in the MS-GNRs \([37]\).

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### 3.7. Determination of cellular uptake of Syndecan—MS-GNRs via TEM

From MSOT tissue phantoms, we found Syndecan-MS-GNRs enhanced OA signal, possibly secondary to the targeting of Syndecan-1 ligands to IGF1-R receptor present on pancreatic cancer cells. In order to determine whether Syndecan-MS-GNRs bound to the surface of pancreatic cancer cells or were internalized into cancer cells, transmission electron microscopy (TEM) was employed. After a 2h incubation, the Syndecan-MS-GNRs were observed to be distributed throughout the cytoplasm, endosome, and nucleus (Figure 5). This image suggests that Syndecan-MS-GNRs were internalized in the cell via an endocytosis process, likely through the formation of flask-shaped...
invaginations that are evident on the cell membrane. The TEM image also shows that the Syndecan-MS-GNRs were enclosed within a vesicular structure (red arrows) lying outside the nucleus. These structures are similar in appearance to early endosomes which generally transport endocytosis materials to lysosomes after maturation. The shape and size of Syndecan-MS-GNRs did not change after being endocytosed in cells as seen in the inset.

The accumulation of Syndecan-MS-GNRs in the nucleus suggests that Syndecan-MS-GNRs were internalized in the nucleus by a receptor-regulated nuclear pore transport mechanism. Syndecan-1 also resides in the cell nucleus, and MS-GNRs (32±2.4 nm length and 19.2±1.59 nm wide) can enter through the nuclear pore via a diffusion process due to MS-GNRs smaller size than the diameter of the nuclear pore complex (20-50 nm) [38, 39].

3.8. Syndecan-MS-GNRs accumulation in vivo as detected using MSOT

Currently, there is no gold-standard for the early detection of pancreatic cancer due to the limitation of traditional imaging techniques and lack of reliable tumor-specific contrast agents. While most of the traditional imaging modalities suffer from poor resolution or shallow depth penetration, multispectral optoacoustic tomography (MSOT) overcomes these limitations by offering microscale resolution combined with reasonable penetration depth [16]. Existing photoacoustic systems have demonstrated adequate resolution with tissue penetration up to 7 cm [40, 41]. Thus, we evaluated detection of pancreatic tumors in vivo by injecting Syndecan-MS-GNRs in orthotopic pancreatic tumor-bearing mice using MSOT. Our results indicate that the combination of Syndecan-MS-GNRs and MSOT adequately detects S2VP10L orthotopic pancreatic tumors (Figure 6), but did not specifically bind to MiaPaca-2 tumors (Fig. S3 in the Electronic Supplementary Material (ESM)).

The biodistribution of Syndecan-MS-GNRs was observed in the mice 4h after tail vein injection of nanoparticles. Serial axial slices were obtained from the liver to the kidney, including the pancreas (Figure 6A). Signal intensity for the Syndecan-MS-GNRs increases through the middle slices, corresponding to the bulk of the tumor mass. Additional orthogonal projections show the 3D structure of the tumor in the xyz-space (Figure 6B). In mice implanted with S2VP10L cells, quantification of the signal intensity indicates increased accumulation of Syndecan-MS-GNRs at the pancreatic tumor site (175.4 MSOT a.u) in comparison to the liver (35.2 MSOT a.u.) (p<0.05) (Figure 6C). Focal accumulation within the pancreatic tumor was not observed in mice implanted with MiaPaca-2 cells (Fig. S3 in the Electronic Supplementary Material (ESM)).
Figure 6: Identification of Syndecan-MS-GNRs in a S2VP10L (positive for IGF1-R) bearing mice using multispectral optoacoustic tomography (MSOT) in vivo. (A) Serial images of a pancreatic tumor mass ranging from 47-51 mm illustrate the capability to precisely define the region of Syndecan-MS-GNRs distribution. (B) Maximum intensity projection of the reconstructed image to determine the 3D conformation of the tumor in xyz-space, enabling measurement of total tumor volume. The pancreatic tumor (PT), blood vessels (BV), liver (L) and kidney (K) are shown. (C) Region of interest analysis demonstrates Syndecan-MS-GNRs accumulation within the tumor (black) and liver (gray dash). The peak accumulation occurred at slice 49.3 mm and contained 175.4 MSOT a.u.

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Calculation of the average Syndecan-MS-GNR signal intensity in the tumor, liver and kidney was performed to compare nanoparticle biodistribution across the IGF1-R positive S2VP10L and IGF1-R negative MiaPaca-2 groups (Figure 7). The Syndecan-MS-GNRs significantly accumulated in the pancreas tumor in S2VP10L mice compared to MiaPaca-2 (233.1 MSOT a.u. vs. 12.4 MSOT a.u.) (p<0.05). Furthermore, minimal off-target binding of Syndecan-MS-GNRs was seen in either the kidney (29.7 MSOT a.u) or the liver (31.4 MSOT a.u) in the S2VP10L group. In MiaPaca-2 mice, substantial renal (114.3 MSOT a.u.) accumulation was observed with minimal accumulation in the liver (35.2 MSOT a.u).

Figure 7: Biodistribution Syndecan-MS-GNRs in S2VP10L (positive for IGF1-R) and MiaPaca-2 (negative for IGF1-R) mouse in vivo by multispectral optoacoustic tomography (MSOT). Biodistribution of Syndecan-MS-GNRs indicates significant accumulation within the pancreas tumor in the S2VP10L (positive for IGF1-R) mice. A region of interest analysis of MSOT images demonstrates that Syndecan-MS-GNRs are significantly accumulated within the pancreas tumor. However, Syndecan-MS-GNRs uptake is minimal in the kidney and the liver in the S2VP10L (positive for IGF1-R) mice, but shows substantial kidney uptake in MiaPaca-2 (negative for IGF1-R). *P < 0.05.
4. Conclusion

Early detection of pancreatic cancer is challenging as tumors may already be metastatic at sizes of 2-3 mm³, on top of the fact that small collections of tumor cells can be dispersed among a thick desmoplastic stroma [42, 43]. To increase the in vivo detection of pancreatic tumors, we utilized a Syndecan-1 ligand to target IGF1-R, which is upregulated in pancreatic cancer cells and tumor stromal cells, but is less expressed in non-malignant tissue [44].

The MSOT offers high potential for the early detection of cancer cells due to hybrid modality, but designing nano-contrast agents for MSOT that provides optimal stability, specificity, safety, and sensitivity is still challenging. Syndecan-MS-GNRs fulfill these criteria as they were stable in biological media and under thermal fluence, demonstrated tumor specificity (Fig. 3, 5, 6 and 7), and were nontoxic [19]. Furthermore, the Syndecan-MS-GNRs were highly sensitive due to a high absorption coefficient and tunability in near infra-red region. MSOT tissue phantoms showed that the Syndecan-MS-GNRs substantially amplified OA signal; almost 10-fold higher than that of Syndecan-PAA-GNRs in S2VP10L cells (Fig. 3B). In vivo, the targeted Syndecan-MS-GNRs demonstrated tumor specific accumulation in IGFR-1 positive tumors, with limited off-target effects. While Syndecan-1 may be a promising ligand for these tumors, future development of additional tumor specific ligands could expand the range of tumor targeted MS-GNRs for use with MSOT.

To the best of our knowledge, this is the first systematic study that compares coating with PAA or MS for GNRs as contrast agents for detection of pancreatic cancer in vivo with MSOT. With continued development, targeted MS-GNRs could serve as multifunctional theranostic platforms, as the MS shell can provide a high surface area for drug payload, while the inner gold nanorods can function as contrast agents for imaging, hyperthermia agents, and allow the light-mediated release of drugs.

Electronic Supplementary Material: Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12274-****.**** (automatically inserted by the publisher). The supplemental data includes: 1) Western blot indicating the status of IGF1-R in cell lines; 2) flow cytometry analysis indicating the ability of a blocking antibody to prevent Syndecan-MS-GNR binding to cells; 3) evaluation of Syndecan-MS-GNR in a MiaPaca-2 orthotopic xenograft mouse model.

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Electronic Supplementary Material

Detection of pancreatic tumors in vivo with tumor targeted mesoporous silica-coated gold nanorods by multispectral optoacoustic tomography

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Supplementary Material

**Fig.1S.** Western blot analysis of S2VP10, and Miapaca-2. Metastatic pancreatic cancer cell lines (S2VP10L) *in vitro* demonstrated up-regulation of IGF1-R compared to non-metastatic pancreatic cancer cell lines (Miapaca-2).

**Fig.2S.** Binding specificity of Syndecan-MS-GNRs and MS-GNRs by flow cytometry in (A) S2VP10L cells

(IGF1-R positive) (B) Blocking agent (anti-IGF1-R antibody) (C) negative control for IGF1-R expression (MiaPaca-2 cells).

Fig. 3S. Identification of Syndecan-1 targeted MS-GNRs in a MiaPaca-2 pancreas tumor using multispectral optoacoustic tomography (MSOT). (A) Serial images of mouse abdomen ranging from pancreas tumor through kidney 47-53 mm illustrate the Syndecan-MS-GNRs distribution in an IGF1-R negative pancreatic cancer model. The IGF1-R negative pancreas tumor does not bind Syndecan-MS-GNR, but these particles accumulate within the kidney. White arrows indicate pancreas tumor location and green arrows indicate the kidneys. (B) Maximum intensity projection of the reconstructed image to determine the 3D conformation of the tumor in xyz-space, enabling visualization of Syndecan-MS-GNR uptake.

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