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Biomedical Applications of Photoacoustic Imaging

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Chemical Engineering

by

Eric Ruike Zhao

Committee in charge:

Professor Jesse Jokerst, Chair
Professor Yi Chen
Professor Liangfang Zhang

2019

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2019

DEDICATION

I dedicate this thesis to my Mom, Dad, Richard Zhao, and Helen Zhao.

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ABSTRACT OF THE THESIS

Biomedical Applications of Photoacoustic Imaging

by

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Photoacoustic imaging has gained significant traction as a pre-clinical imaging modality in the last few decades. Combining the spectral sensitivity of optical imaging and the spatial and temporal resolution of ultrasound, photoacoustic imaging is non-invasive and safe due to the use of non-ionizing radiation in the near-infrared range. Many strategies have been developed to improve image contrast and treatment of diseases for diagnostic and therapeutic purposes. First, a brief and surface-level overview of photoacoustic

imaging, its function, theoretical considerations, and potential applications are discussed. Then, the ability of silicon carbide nanoparticles as a multimodal contrast agent for stem cell imaging is shown. It has been previously reported that silicon carbide particles are photoluminescent due to quantum confinement effects and have been reported in imaging different cell lines. However, reliance on optical methods suffers from limited penetration depth for *in vivo* tracking. In addition to the photoluminescent property of silicon carbide, we show for the first time that they are also capable of being applied to photoacoustic imaging. These durable particles are biocompatible with mesenchymal stem cells and are capable of long-term cell tracking through photoluminescence *in vitro* and through photoacoustic imaging *in vivo*. The improved penetration depth of photoacoustic imaging provides additional versatility to silicon carbide nanoparticles as an *in vivo* contrast agent.

CHAPTER 1

Photoacoustic Imaging for Medical Diagnosis and Treatment

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1.1 Introduction

Ultrasound imaging is the most commonly used modality in the clinic after X-ray imaging.¹ Sound pulses generated in the megahertz frequency range are emitted by a transducer and reflected depending on the impedance mismatch between tissues to generate contrast. Contrast is also determined by the contributions of refraction, absorption, and scattering of the sound waves. In other words, this modality is entirely dependent on the mechanical properties of the body. While a major advantage of ultrasound is high imaging depth and temporal resolution, it lacks the ability to distinguish between spectrally unique structures. Tissues that have the same mechanical properties are difficult to distinguish even if they have entirely different optical absorption properties. Although optical methods provide improved contrast, images are restricted to surface features due to the limited penetration depth and high absorption of light in the visible region by endogenous chromophores.^{2,3} On the other hand, near-infrared (NIR) light has a significantly improved penetration depth of a few centimeters compared to visible light.⁴ Hence, imaging modalities that utilize this range are capable of imaging at greater depths.

Photoacoustic imaging is a non-invasive hybrid modality that bridges the gap between optical and ultrasound imaging. While optical imaging relies on the interaction of light with matter and ultrasound relies on the interaction of sound with matter, photoacoustic imaging combines both. Most simply, it is described as a ‘light in, sound out’ method (**Figure 1.1**). Nanosecond laser pulses in the NIR range are used to illuminate an area of interest. The energy is absorbed, converted into heat, and causes photothermal expansion and contraction of the tissue.^{5,6} This is known as the photoacoustic effect. The

generated sound waves that can then be detected through an ultrasound transducer and processed into an image. Furthermore, acoustic waves are scattered far less than light in tissue,⁵ which improves penetration depth of photoacoustic imaging since light does not need to travel back out of tissue. One of the main advantages of photoacoustic imaging is the ability to tune the wavelength of excitation in the laser. This allows for selective absorption of the chromophore or contrast agent of interest to minimize noise from background tissue. Alternatively, exogenous small molecule dyes or nanomaterials can be engineered such that the maximum absorption is in the NIR range. These are designed to have various diagnostic and therapeutic benefits.

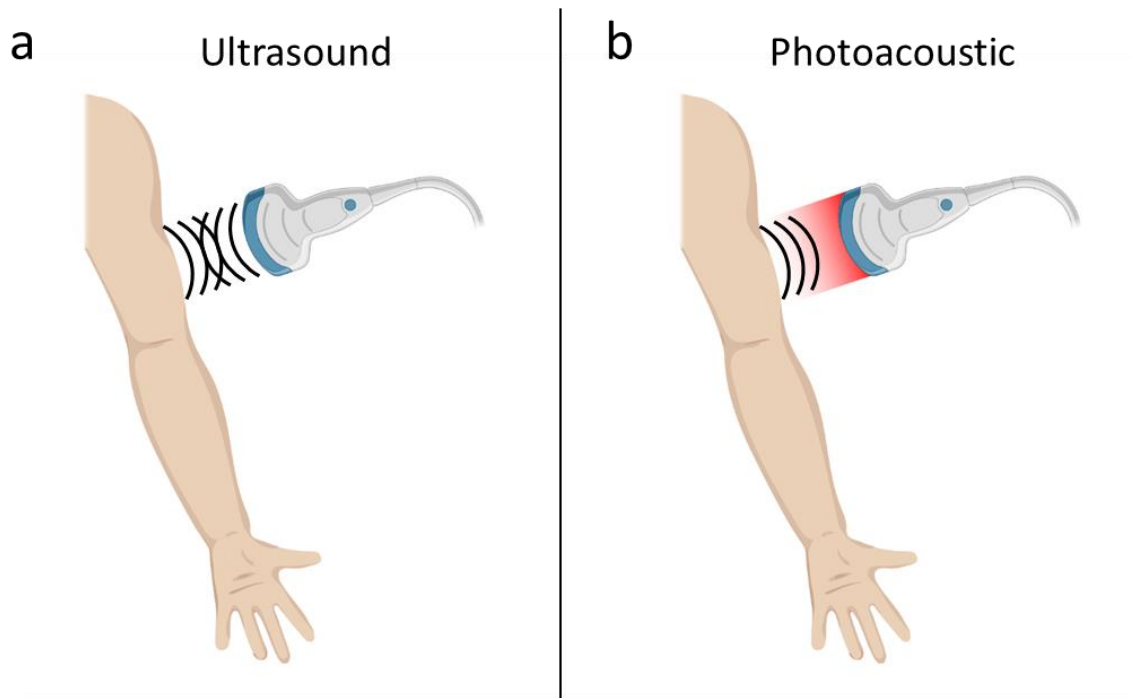


Figure 1.1. Schematic representation of ultrasound and photoacoustic imaging modalities. (a) In ultrasound imaging, high frequency sound pulses are generated, reflected by the body, and detected. (b) In photoacoustic imaging, laser pulses cause rapid thermal expansion and contraction, generating acoustic waves that can be detected.

1.2 Theory

The intensity of a photoacoustic signal is dependent on the amplitude and distribution of the pressure waves generated by the sample. In brief, the generated pressure wave, p_0 , as a function of position can be described by the equation

$$p_0(r) = \Gamma \mu_a(r) \phi(r; \mu_a, \mu_s, g)$$

where Γ is the Grüneisen parameter, μ_a is the absorption coefficient, ϕ is the optical fluence, μ_s is the scattering coefficient, and g is the anisotropy factor.⁷ More simply, the relation can be split into parameters that define the sample's mechanical properties and optical properties. The Grüneisen parameter is dependent on the properties of the tissue and is defined as $\Gamma = \beta c^2 / C_p$, where β is the volume thermal expansivity, c is the speed of sound, and C_p is the constant pressure heat capacity. This dimensionless quantity describes the conversion of light absorption to pressure and is similarly described as the efficiency of energy transfer. The pressure wave amplitude is also largely dependent on how strongly the sample absorbs light and the intensity of the light source.

Quantification of chromophores with photoacoustic imaging *in vivo* is extremely difficult. In optical spectroscopy, the absorption of a chromophore at each wavelength can be easily quantified through a linear relationship. Assuming that the medium is homogenous and has known optical properties, the chromophore's concentration can be determined. This assumption breaks down with photoacoustic imaging *in vivo*. The main reason is due to the nonlinearity of light fluence from the scattering and absorption effects of various tissue.⁸ As a result, a chromophore lying within the tissue will not experience the same optical fluence as the surface of the tissue. A simpler example illustrating this

concept involves two spectrally unique chromophores stacked on top of each other at different depths. The absorption characteristics of the first chromophore will affect the light fluence that excites the second chromophore, additionally affecting the generated photoacoustic signal. In other words, wavelengths that are strongly absorbed by the first will minimize the amount of light that reaches the second. By extension, this effect is far more complicated in the body due to the heterogeneity of surrounding tissue. The photoacoustic spectrum of a specific point is not the same as the linear combination of absorption spectra of the chromophores at that point.⁹ The difficulty of quantifying concentrations is an important limitation of photoacoustic imaging, but a success in doing so would have major implications. The ability to non-invasively determine the exact concentration of a circulating drug or nanomaterial in the body would prevent the need for invasive blood sampling or surgery. Additional theory and the implications of photoacoustic quantification are explained in great detail elsewhere.⁷⁻¹⁰

1.3 Photoacoustic contrast agents

1.3.1 Endogenous chromophores

There are a wide variety of NIR-absorbing contrast agents that either exist naturally within the body or have been developed artificially. For endogenously existing chromophores, the more commonly imaged chromophores include hemoglobin and melanin. Hemoglobin and its alternate forms—such as oxyhemoglobin, deoxyhemoglobin, methemoglobin, and carboxyhemoglobin—have varying absorption spectra¹¹ and can be exploited for contrast in photoacoustic imaging. In particular, the differences between

oxyhemoglobin and deoxyhemoglobin allow for the measurement of oxygen saturation based on a ratiometric analysis. In theory, only two wavelengths are needed to determine the relative concentrations of each, but it is beneficial to use more in order to minimize measurement errors.¹²

Recently, Okumura et al. measured the oxygen saturation in ischemia-induced acute kidney injured mice.¹³ They found a significant difference between mice with mild and severe injuries 24 hours after ischemia-reperfusion surgery, indicating that the severity of kidney ischemia could be non-invasively monitored with sufficient sensitivity. Another potential application demonstrated by Wood et al. involves monitoring oxygen saturation throughout the length of mouse femurs with acute lymphoblastic leukemia.¹⁴ A phenotype of this cancer is the creation of hypoxic niches within the bone marrow. Not only do they demonstrate that oxygen saturation can be measured through bone, but they also found that diseased mice show significantly lower oxygen levels. They also found a greater variation of hypoxia along the femur within these mice.

Melanin has a strong and broad absorption profile in the NIR range that can be exploited for visualizing the thickness of melanoma tumors¹⁵ and metastases within sentinel lymph nodes.¹⁶ A study conducted in mice demonstrated that varying stages in melanoma development could be easily distinguished.¹⁵ Both ultrasound and photoacoustic images were able to clearly track the size over time. The tumor size was measured photoacoustically and grew from a thickness of less than 0.5 mm on day 7 compared to 2.51 mm on day 30. Furthermore, additional angiogenic blood vessels could be visualized. In a different study, metastatic melanoma were visualized in human sentinel lymph nodes

with a significantly higher success rate compared to the protocols established by the European Organisation for Research and Treatment of Cancer.¹⁶ Out of 148 imaged lymph nodes, all that contained metastatic tumors were properly identified as suspicious and were later confirmed by histology. None of the metastatic lymph nodes were improperly diagnosed as a false-negative, further implying the sensitivity of photoacoustic imaging to detect melanoma.

Although photoacoustic imaging is capable of detecting specific endogenous molecules and structures, it is limited from detecting other diseases that contain no inherent contrast. Many diagnoses are inconclusive without additional signal amplification. Thus, strategies have been developed to improve contrast and simultaneously treat diseases with the help of external agents. Many varieties of molecular and nanomaterial-based contrast agents have been utilized, but they must be able to overcome the background signal of these endogenous contrast agents.

1.3.2 Exogenous molecules and nanomaterials

Small molecules or nanomaterials are more commonly employed as contrast agents since they can be tuned to target specific tissues, track cells, or even activate under specific stimuli. These contrast agents must satisfy certain criteria in order to be employed in conjunction with photoacoustic imaging. They would ideally possess the following qualities: a narrow absorption peak for specificity, high absorption coefficient to minimize the limit of detection, low quantum yield for efficient non-radiative decay of energy, high

photostability for recurrent and long-term imaging, and biocompatibility to avoid any potential side effects.

Commonly used NIR-absorbing dyes for photoacoustic imaging include indocyanine green (ICG) and methylene blue, which have both been approved by the U.S. Food and Drug Administration (FDA) for clinical use. ICG has been applied for imaging tumors with leaky vasculature,¹⁷ rat brains,¹⁸ and sentinel lymph nodes in both rats¹⁹ and humans.¹⁶ Methylene blue is commonly used to treat methemoglobinemia,²⁰ but has been applied to photoacoustic imaging for measuring dissolved oxygen content²¹ and heparin concentration in blood.²² Small-molecule dyes can be advantageous due to their biocompatibility and accessibility, but also suffer from nonspecific signal enhancement without the help of a nanocarrier.²³

Complex molecules have been designed to be more specific for targeting purposes. A probe was designed by Levi et al. that contained two chromophores that were photoacoustically active at two unique wavelengths.²⁴ BHQ3 and Alexa750 were coupled together through a peptide sequence that is cleavable by matrix metalloprotease-2 (MMP-2). Before cleavage, the probe showed high photoacoustic signal at both 675 nm and 750 nm. Taking the difference in the respective signal intensities of each wavelength would result in a silent probe. After cleavage, the BHQ3 portion was left with a cell-penetrating peptide that accumulated within cells, while the Alexa750 portion was washed away. The targeted cells showed significantly brighter signal than a control probe that was uncleavable.

Nanomaterials are a significant portion of contrast agents that are creatively engineered for a variety of functions. In addition to enhancing the signal-to-noise ratio they can also be designed to target a specific molecule or tissue, activate under exposure to stimuli, encapsulate and control the release of drugs, or label and track cells.

Nanoparticles are beneficial to use for targeted diagnostics due to the large surface area available for surface functionalization. Attaching additional moieties improves the probability of successfully binding the target.²³ A common tumor targeting strategy involves functionalizing a nanoparticle surface with the tripeptide arginine-glycine-aspartic acid (RGD). This peptide sequence binds to $\alpha_v\beta_3$ integrin found on angiogenic vessels.²⁵ Cheng et al. developed nano-sized gold tripods functionalized with a cyclic RGD to image a glioblastoma model through photoacoustic imaging.²⁶ The anisotropic nanoparticles were less than 20 nm in size and showed a maximum photoacoustic signal in the tumor only 2 hours after intravenous injection. In another unique targeting strategy, Wu et al. created silver sulfide nanoprobe conjugated with ICG and amphipathic C18/PEG polymers to target atherosclerotic plaques.²⁷ The nanoprobe in atherosclerotic mice showed a 6-fold increase in photoacoustic signal 3 hours after injection compared to pre-injection images. A peak signal intensity also remained for 24 hours, providing a long window for potential imaging. Accumulation occurred due to the lipophilicity of the C18 chain on the nanoprobe. This was controlled by injecting ICG only in a separate mouse, which showed no signal change throughout the entire imaging period.

The versatility of nanoparticles can be extended to create multifunctional systems that combine imaging and therapeutic concepts. Jung et al. created a nanoparticle that

photoacoustically activates while simultaneously releasing antioxidant and anti-inflammatory molecules upon exposure to a hydrogen peroxide-rich thrombus.²⁸ The nanoparticles were made by conjugating borylbenzyl carbonate with a fluorescent IR780 to maltodextrin. Introduction of hydrogen peroxide resulted in the formation of CO₂ bubbles that were capable of amplifying both ultrasound and photoacoustic signals. Furthermore, oxidation of the aryl boronate group generated hydroxybenzyl alcohol for antioxidant treatment. In order to specifically target thrombi, they additionally conjugated a lipopeptide with a high affinity to fibrin to the surface. Particles injected into mice showed a gradual increase in signal up until 30 minutes post-injection. In addition, histological staining showed significantly less thrombus formation compared to non-specific nanoparticles, indicating the efficacy of the targeting group. This design not only allowed for non-invasive visualization of the thrombus, but also acted as a hydrogen peroxide scavenger and anti-inflammatory agent.

The larger size and improved stability of nanoparticles in comparison to small molecules allows them to be applied for cell tracking applications. Assuming appropriate biocompatibility, cells can endocytose nanoparticles and retain them for an extended period of time. A trimodal contrast agent for stem cell tracking was developed by Lemaster et al. for photoacoustic imaging, magnetic particle imaging (MPI), and ultrasound.²⁹ The poly(lactic-co-glycolic acid) (PLGA) nanobubbles were loaded with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) and iron oxide to facilitate photoacoustic and MPI contrast, respectively. In another example, Kim et al. developed a Prussian blue nanocomplex for photoacoustic contrast and conjugated it with poly-L-lysine

for improved cell uptake.³⁰ Without poly-L-lysine, a very high negative zeta potential limited cellular uptake due to repulsion from the negatively charged membrane. After conjugation, the zeta potential was highly positive and cell labeling capacity. They were able to image 50,000 labeled mesenchymal stem cells subcutaneously and track them over a period of 14 days.

The high versatility of nanomaterials exists due to the large choice in material, size, shape, conjugating ligands, and molecular probes. There are many review articles that further highlight various molecular probes, nanomaterial classes, and their respective advantages in photoacoustic imaging.^{2, 23, 31-34}

1.4 Outlook and potential for clinical translation

While many studies and strategies have shown the potential of photoacoustic imaging, additional work is needed to further verify the safety and efficacy of various contrast agents. For the same reason why novel nanomaterials or probes have been continuously produced, there is still room for further biocompatibility and characterization. Long-term toxicity is a major concern as many studies are limited by the need for animal sacrifice for histological staining. Novel designs also lack the follow-up research needed to prove that the work is reproducible, limiting the potential for further improvement and translation. Nanoparticles can be difficult to purify, quantify, and synthesize homogeneously.³⁵ This can be problematic if there is any potential for large-scale manufacturing.

Despite the additional studies needed for contrast agent design, photoacoustic imaging as a field is still rapidly growing. More recent work shows the use of an LED-based photoacoustic system, which is cheap, portable, and uses lower energy light.³⁶ Furthermore, while this review focuses on the applications within the first NIR window, it is also possible to use higher wavelength light in the second NIR window to further improve penetration depth. The most recent research is nicely summarized by Huang et al.³⁷

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CHAPTER 2

Silicon Carbide Nanoparticles as a Photoacoustic and Photoluminescent Dual-Imaging Contrast Agent for Long-Term Cell Tracking

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Abstract

Silicon carbide nanoparticles (SiCNPs) are durable, physically resilient, chemically inert, and biocompatible. Silicon carbide particles smaller than 10 nm show photoluminescence due to quantum confinement effects and have been reported in imaging different cell lines. To further explore the potential of silicon carbide nanomaterials in cell imaging, we studied the photoluminescence and photoacoustic properties of three SiCNPs of approximately 30, 80, and 620 nm. All these SiCNPs show photoluminescence and photoacoustic signals; and the 620 nm silicon carbide nanoparticles (SiCNP620) show the highest photoluminescence and photoacoustic intensity. The SiCNP620 are biocompatible with good cell labeling capacity. They could image mesenchymal stem cells *in vitro* for more than 20 days via photoluminescence even when the cells were differentiated into adipocytes and osteocytes. The same SiCNP620 could also produce photoacoustic signals and track stem cells *in vivo* for over 14 days.

2.1 Introduction

Silicon carbide (SiC) materials are used as dental implants, bone prosthetics, coronary heart stents, and brain-machine interfaces devices.¹ Bulk silicon carbide materials are biocompatible, durable, resilient, and inert. Thus, they are often used as coatings for biomedical devices to prevent leakage of ions and/or reduce biofouling.² The emergence of silicon carbide nanomaterials two decades ago has resulted in several novel biomedical applications like cell imaging.³

Silicon carbide quantum dots have been used as photoluminescent probes for cell imaging. For silicon carbide, the word photoluminescence more accurately describes the luminescent property than fluorescence due to the delayed emission time.⁴ Silicon carbide quantum dots have strong photoluminescence due to their highly improved radiative recombination rates in such small clusters—only few nanometers. Botsoa *et al.* reported using silicon carbide quantum dots with a cubic symmetry crystalline structure (β -SiC) to image living 3T3-L1 fibroblast cells via fluorescence microscopy.⁵ Fan *et al.* synthesized β -SiC quantum dots via electrochemical etching and used these quantum dots to label human fetal osteoblast cells.⁶ Zakharko *et al.* demonstrated that the silicon carbide quantum dots could label fibroblast cells and the photoluminescence of these quantum dot-labeled cells could be greatly enhanced via localized plasmons.⁷ Beke *et al.* showed that silicon carbide quantum dots made by chemical etching could image live neuron cells via two-photon microscopy.⁸

In addition, sub-micron silicon carbide nanoparticles (SiCNPs) could also enter and visualize cells via nonlinear photoluminescence signals.^{9, 10} For example, Rogov *et al.*

labeled 3T3-L1 fibroblast cells with 3C-SiC nanoparticles larger than 100 nm and then imaged the labeled cells with nonlinear microscopy.⁹ Boksebeld *et al.* used folate-modified 3C-SiC nanoparticles with a diameter of 150 nm to label cancer cells *via* multi-photon microscope.¹⁰ The non-centrosymmetric crystalline structure and relatively large diameter (associated with a large two-photon absorption cross-section) of SiC nanoparticles provided an intense emission peak. Previously, we demonstrated the ability of silicon carbide nanoparticles of approximately 80 nm and 600 nm to track human mesenchymal stem cells (hMSCs) via photoluminescence.¹¹

While many studies have shown the utility of silicon carbide nanoparticles for *in vitro* cell imaging, there is little evidence that these nanoparticles can be used for *in vivo* cell tracking—perhaps because photoluminescence has poor tissue penetration depth.¹² This poor penetration of photons limits the use of silicon carbide nanomaterials in cell therapy applications that require *in vivo* tracking of transplanted cells. Photoacoustic imaging is an emerging technique combining the contrast and spectral tuning of optical imaging and high temporal and spatial resolution of acoustic imaging.^{13, 14} The improved penetration depth of photoacoustic imaging over optical and fluorescent imaging gives it an advantage for *in vivo* systems.¹⁵ Here, we show for the first time that the silicon carbide nanoparticles generate photoacoustic signals and can track cells *in vivo*.

In this work, we used silicon carbide nanoparticles to track mesenchymal stem cells. Mesenchymal stem cells are a promising regenerative medicine but their retention is poor.¹⁶ It is important to track long-term the injected cells—including the cell location, cell numbers, and cell fate.¹⁷ Silicon carbide nanoparticles have great potential in tracking

mesenchymal stem cells due to their biocompatibility.¹¹ Here, we compared the photoluminescence and photoacoustic intensities of silicon carbide nanoparticles with three sizes. Then, we used the one with the strongest photoluminescence and photoacoustic intensities to label and track stem cells. This nanoparticle can image mesenchymal stem cells *in vitro* via fluorescence microscopy and, moreover, track stem cells *in vivo* via photoacoustic imaging. Both the photoluminescence and photoacoustic signals of this nanoparticle in mesenchymal stem cells are stable for over 10 days. The photoluminescence of this silicon carbide nanoparticle in mesenchymal stem cells were seen even when the cells differentiated to adipocytes and osteocytes.

2.2 Materials and Methods

Nanoparticle characterization

Silicon carbide nanoparticles of different sizes were purchased from US Research Nanomaterials Inc. (US2161, US2022, and US2011) and were calcined at 600°C for 2 hours before use as described previously.¹¹

Particle sizes were analyzed with TEM images that were taken on a JEOL1400-Plus with a Gatan Orius 600 camera. High resolution TEM (HRTEM) images were obtained via a FEI Tecnai F20 operated at 200 kV, and the element mapping was performed in this electron microscope in STEM mode. STEM images and EDX spectra were taken on a HD-2000 STEM (Hitachi) and Quantax EDS (Bruker) at an accelerating voltage of 200 kV. X-ray diffraction (XRD) patterns were scanned on a Rigaku Miniflex XRD unit operating at 40 kV and 40 mA using Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$) with a 2θ step size of 0.02° within $20\text{-}80^\circ$. Zeta potential was measured via a Malvern Zetasizer ZS90.

Absorbance spectra of SiCNPs (20 $\mu\text{g/mL}$) were obtained with a UV-vis microplate reader (SpectraMax M5, Molecular Devices). Photoluminescence spectra of SiCNPs suspended in water (20 $\mu\text{g/mL}$) were examined with the same UV-vis microplate reader under the time-resolved fluorescence mode. To maximize the signal intensity, the integration time was set to 1.5 ms and 100 flashes were recorded per read. Photoluminescence spectra were also recorded via a customized fiber-coupled spectrometer (Princeton Instruments). A layer of SiC nanoparticles were added on glass slide, dried, and then excited by 325 nm or 442 nm UV light (~ 10 mW) at an angle of $\sim 45^\circ$ relative to the sample plane. The emission spectra were collected through a 20x objective and recorded by the fiber-coupled spectrometer. A 450 nm long pass filter was applied in front of the spectrometer when the particles were excited by 442 nm light using the same optical setup laser.

Photoacoustic scans were obtained using a Vevo LAZR from Visualsonics.¹² SiCNP samples were resuspended in water at a concentration of 1 mg/mL. Samples were then loaded into polythene tubing and scanned with an array photoacoustic transducer (LZ250, Visualsonics, Inc.) operating at 25 MHz center frequency. The photoacoustic spectra between 680 nm and 970 nm were scanned with a step size of 5 nm. To determine the limit of detection by photoacoustic imaging, SiCNPs were resuspended in water at different concentrations (100, 50, 25, 10, 5, and 0 $\mu\text{g/mL}$) and scanned with a wavelength at 725 nm. The limit of detection was defined as the concentration that had a signal intensity at 3 standards deviations above the mean.

Cell culture

Human mesenchymal stem cells (hMSCs, cat #: PT-2501) and cell culture media (cat #: PT-3001) were purchased from Lonza. Human MSCs from passage 2-10 were used. Mouse mesenchymal stem cells (mMSCs, cat #: MUBMX-01001) were purchased from Cyagen. Mouse MSCs were used between passages 8-12 and were cultured with Dulbecco's Modified Eagle Medium (DMEM) from Gibco supplemented with 10% fetal bovine serum (Sigma) and 1% antibiotic-antimycotic (Thermo Fisher Scientific). Cell media was replaced every 2-3 days. Cells were grown to 80-90% confluency. For passage and usage, cells were detached with 0.25% Trypsin-EDTA (Gibco), then centrifuged at 1,000 rpm for 5 minutes, resuspended, and counted by hemocytometer.

For labeling condition studies, hMSCs were plated into a 6-well plate and grown until fully confluent. Then, 2 mL of SiCNPs suspensions in cell media at concentrations of 0, 50, 100, and 200 $\mu\text{g/mL}$ were added to each well separately. After a specific incubation time (1, 4, or 8 hours), cells were washed with PBS thrice to remove free nanoparticles and then collected. The cell labeling capacity was then quantified by thermogravimetric analysis (TGA) and photoacoustic imaging independently. TGA experiments were performed with approximately 400,000 cells resuspended in 20 μL of Millipore water using a Perkin-Elmer STA 6000 Simultaneous Thermal Analyzer. The labeled cells were heated from 25 to 600°C at a heating speed of 10°C/min and then held at 600°C for 2 hours. SiCNPs were also analyzed by TGA as a positive control. For photoacoustic imaging, labeled cells were dispersed in a 1:1 warm mixture of PBS: agarose (1%), added in to polyethylene tubes, cooled, and then scanned.

The cytotoxicity of SiCNP620 to mesenchymal stem cells was studied by plating cells into a 96-well plate at a density of 5,000 cells/well. Cells were incubated with SiCNP620 suspensions at different particle concentrations—400, 200, 100, 50, 25, and 0 $\mu\text{g/mL}$. Positive controls included 400 $\mu\text{g/mL}$ SiCNP600 and media only. Eight replicates were tested. After 48 hours, an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega) assay was performed by adding 100 μL of a 1:10 v/v MTS:media solution to each well. The cells with MTS reagent were incubated for four hours. Next, to avoid any interferences of nanoparticles on the absorbance, 70 μL of supernatants from each well were transferred to a new plate, and the absorbance was read at a wavelength of 490 nm.

The effect of SiCNP620 on the proliferation of mesenchymal stem cells was also studied. Cells were plated in a 96-well plate at a density of 3,000 cells/well and then incubated with SiCNP620 suspensions at different particle concentrations—400, 200, 100, 50, 25, and 0 $\mu\text{g/mL}$ for different durations—1, 2, 3, and 4 days. Positive controls included 200 $\mu\text{g/mL}$ SiCNP600 and media only. Eight replicates were tested for each concentration and duration. At each time point, a Resazurin (Sigma-Aldrich) assay was performed by adding 100 μL of a 1:10 v/v Resazurin:media solution to each well, followed by incubation for 4 hours. We then transferred 70 μL of the supernatant from each well to clean wells and read the fluorescence at an excitation of 550 nm and emission of 585 nm.

In vitro photoluminescence imaging of cells

Photoluminescence imaging was conducted *via* EVOS fluorescence microscope with a Texas Red channel, which corresponds most closely to the emission wavelength of

SiC. SiCNP620-labeled (50 $\mu\text{g/mL}$ of SiCNP620, incubated 4 hours) and unlabeled cells were plated separately in a 6-well plate. Cell morphology was then recorded with photoluminescence imaging on different days for three weeks. Cells plated in other wells were treated with adipogenic or osteogenic induction media for three weeks. The morphology changes were recorded with bright field and photoluminescence imaging.

In vivo cell tracking by photoacoustic imaging

All animal studies were carried out in accordance with the regulations set by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego. Unlabeled mMSCs and SiCNP620-labeled mMSCs were detached, counted, resuspended in PBS, and kept in ice. Subcutaneous injections were performed by mixing the cell solution and cold Matrigel (Corning) at a 1:1 v/v ratio. Then, 100 μL of the mixture was injected subcutaneously into the mouse and immediately scanned with a photoacoustic transducer (LZ250, Visualsonics, Inc.) operating at 25 MHz center frequency. The photoacoustic scans were also performed 3, 7, and 14 days after the injections.

2.3 Results and Discussion

Silicon carbide nanoparticles with three different sizes were synthesized via plasma chemical vapor deposition by US Research Nanomaterials, Inc (US2011, US2022, and US2161). The average size of these nanoparticles was 33 ± 12 nm, 78 ± 16 nm, and 624 ± 94 nm based on their TEM images (**Figure 2.1a-d**), which were subsequently labeled as SiCNP30, SiCNP80, and SiCNP620. Additionally, SiCNP30 and SiCNP80 are more circular than SiCNP620: the circularity of SiCNP30, SiCNP80, and SiCNP620 were 0.90

± 0.03 , 0.89 ± 0.13 , and 0.61 ± 0.09 . All three SiCNPs were negatively charged in water, where the pH lies above the isoelectric point of 3C-SiC.¹⁸ The zeta potentials of the nanoparticles from smallest to largest were -19.0, -25.3, and -30.7 mV. According to Chung *et al.*, the differences in zeta potential of these SiCNPs would have insignificant effects on their cell uptake.¹⁹

We characterized the crystal structure of these nanoparticles in more detail to confirm that these particles have similar crystalline structure. The fast Fourier transform patterns in the high resolution TEM images are typical diffraction patterns for a cubic crystal structure along the [110] zone axis. The measured lattice spacing of the (111) plane for the three nanoparticles are 2.54Å, 2.58Å and 2.57Å, respectively (**Figure 2.1e-g**). Those measured spacings are close to the standard values of powder diffraction file card 96-900-8857. X-ray diffraction spectra of all SiCNPs show peaks around 2θ 35.77°, 41.54°, 60.2°, 72.04°, and 75.79°, which corresponds to the SiC(111), SiC(200), SiC(220), SiC(311), and SiC(222) peaks of cubic 3C-SiC (PDF 96-901-8857) (**Figure 2.1h**). 3C-SiC, or β -SiC polytype, is the most thermodynamically stable polytype and has the lowest thermal conductivity.²⁰ The other peaks match with SiO₂ peaks (powder diffraction file card 96-412-4080, 96-900-0779, 96-901-4487) and are likely due to the glass sample holder.

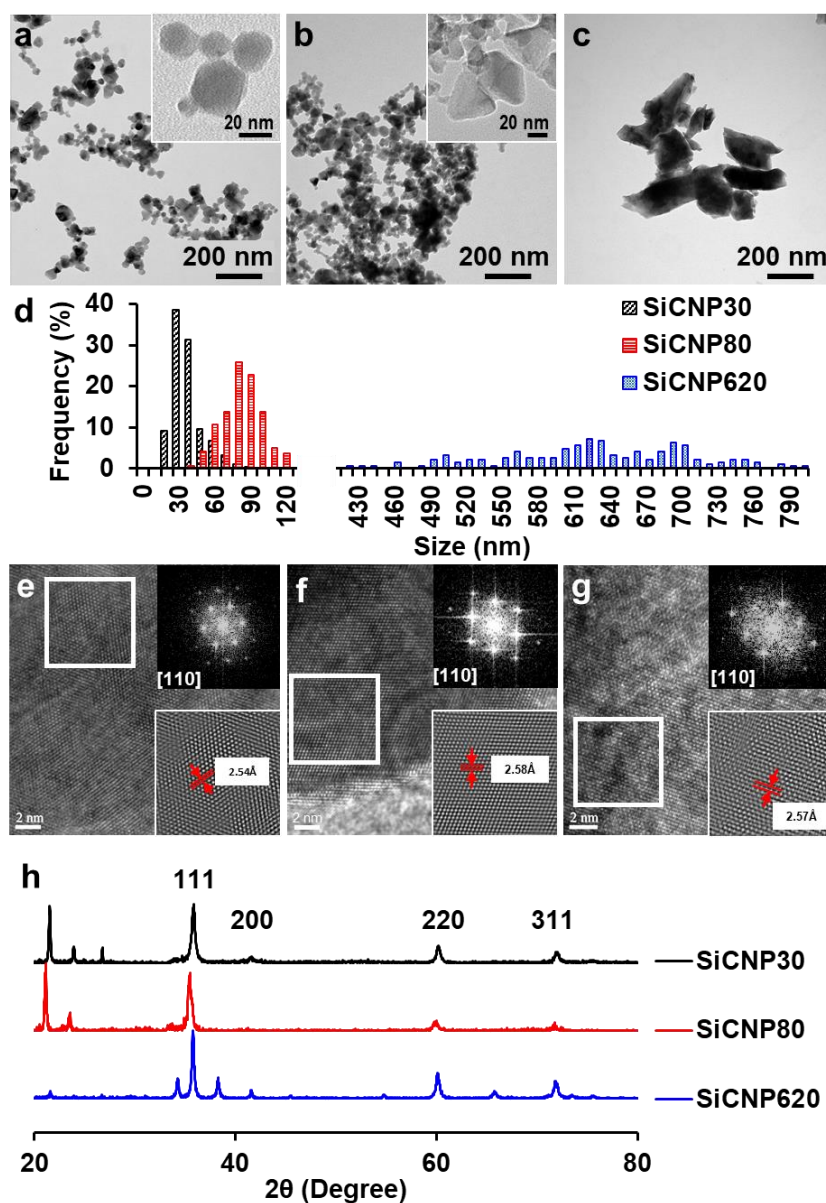


Figure 2.1. TEM images of SiCNPs with average sizes of (a) 30 nm, (b) 80 nm, and (c) 620 nm. The circularity of SiCNP30, SiCNP80, and SiCNP620 were 0.90 ± 0.03 , 0.89 ± 0.13 , and 0.61 ± 0.09 . (d) TEM size distributions of SiCNPs. High resolution TEM images and fast Fourier transformation patterns of (e) SiCNP30, (f) SiCNP80, and (g) SiCNP620 show a typical diffraction pattern of a cubic crystal structure along the [110] zone axis. The measured lattice spacing of the (111) plane for the three types of silicon carbide particles are 2.54Å, 2.58Å and 2.57Å, respectively. (h) X-ray diffraction spectra of all SiCNPs show peaks around 2θ of 35.77° , 41.54° , 60.2° , and 72.04° , which corresponds to the SiC(111), SiC(200), SiC(220), and SiC(311) peaks of cubic 3C-SiC (powder diffraction file card 96-901-8857).

After confirming the crystal structure of these particles, we investigated the effect of size on light absorption and photoluminescence. SiCNPs (20 $\mu\text{g/mL}$) showed size-dependent light absorption (**Figure 2.2a**). The absorbance spectra of SiCNP30 and SiCNP80 showed that their absorbance decreased with increasing wavelength, which are similar to the absorbance of 3C-SiC nanocrystals smaller than 10 nm. The sharper decrease in absorbance of SiCNP30 than SiCNP80 may indicate more quantum-confinement events in the smaller SiCNPs.⁶ Remarkably, SiCNP620 has a very broad absorbance peak at 560 nm (2.2 eV) corresponding to the bandgap of bulk 3C-SiC. The increased absorbance intensity of SiCNP30 and SiCNP80 in the short wavelength range may be due to the high contribution of Rayleigh scattering for smaller particles. The scattering effect diminishes with increasing wavelength, explaining the gradual decline in absorbance intensity.

The photoluminescence of all SiCNPs were examined at varying excitation and emission conditions. The excitation spectra of 2 mg/mL SiCNPs in water were studied at emission 540 nm with a UV-vis microplate reader (SpectraMax M5, Molecular Devices). The excitation peaks of SiCNP30, SiCNP80, and SiCNP620 were around 280 nm, and the intensity increased with increasing particle size (**Figure 2.2b**). The emission spectra of these SiCNPs suspensions were scanned with excitations at 270, 280, 300, and 350 nm (**Figure 2.2c**). SiCNP620 showed the highest photoluminescence intensity among all SiCNPs. The photoluminescence of SiCNP620 was approximately 2-fold of SiCNP80 and 8-fold of SiCNP30 when the excitation wavelength was 280 nm. The emission spectra of dry SiCNPs were also studied with a 10-mW laser (325 nm). The emission peaks of SiCNP30, SiCNP80, and SiCNP620 were 538 nm (2.3 eV), 568 nm (2.2 nm), and 614 nm

(2.0 eV), respectively (**Figure 2.2d**), indicating a red-shift of photoluminescence as the particle size increases.

The photoluminescence mechanisms of silicon carbide nanoparticles is complicated and remain controversial.²¹ The photoluminescence of silicon carbide nanoparticles can be linear and non-linear. The linear photoluminescence may be produced by quantum confinement effect in the small features²² or the amorphous fractions of silicon carbide nanoparticles and the Si-O bonds on the surfaces. This might be one reason for the stronger photoluminescence of SiCNP620 than SiCNP30 and SiCNP80, because SiCNP620 showed the lowest circularity—0.6 compared to 0.9 of SiCNP 30 and SiCNP80. Second, the non-linear photoluminescence can be second-harmonic generation or two-photon excitation fluorescence (photoluminescence is used in this paper because it more accurately describes the luminescent property than fluorescence due to the delayed emission time.^{9, 10} A larger diameter is favored for the non-linear photoluminescence because the two-photon absorption cross-section is larger.¹⁰ This might be another reason for the strongest photoluminescence of SiCNP620 among the three nanoparticles.

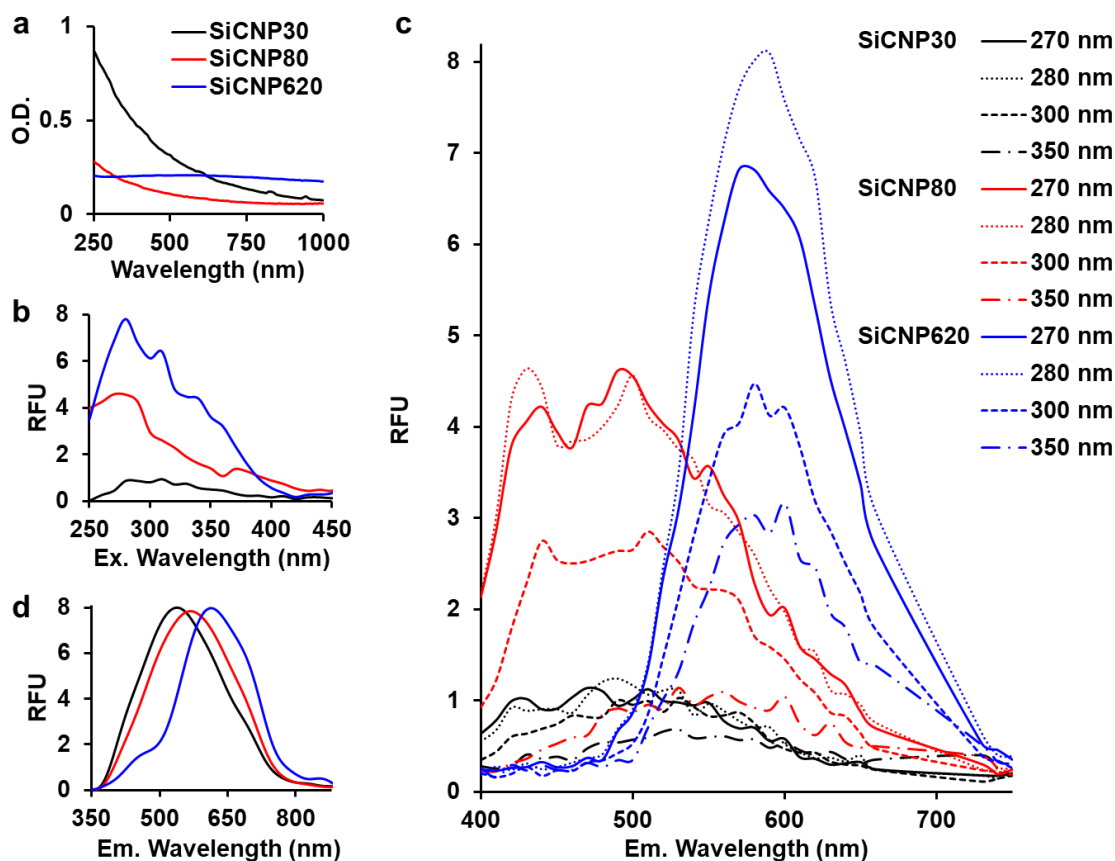


Figure 2.2. Optical properties of all SiCNPs. (a) Absorbance spectra of SiCNP suspensions. (b) Excitation spectra (emission 540 nm) and (c) emission spectra at varying excitation wavelengths of SiCNP suspensions. (d) Emission spectra of dry SiCNPs using a laser excitation source of 325 nm. The intensities were normalized. Black, red, and blue represent SiCNP30, SiCNP80, and SiCNP620, respectively.

Next, we compared the photoacoustic properties of these silicon carbide nanoparticles. The photoacoustic spectra show that all particles had a broad wavelength signal at a concentration of 1 mg/mL in water (**Figure 2.3a**). The nanoparticles were then scanned at a fixed wavelength of 725 nm, and SiCNP620 had the highest photoacoustic intensity (**Figure 2.3b**). The photoacoustic intensity of SiCNP30 and SiCNP80 were 23% and 82% of that of SiCNP620, respectively (**Figure 2.3c**). Hence, we conclude that the

photoacoustic intensity increased with increasing particle size. This is expected because the absorbance of near-infrared light by SiCNP620 was higher than both SiCNP30 and SiCNP80 (**Figure 2.2a**) and particles with a larger cross-section, provided that the material and light fluence are the same, will have a greater photoacoustic signal.^{23, 24} Interestingly, the largest nanoparticle had both the strongest photoluminescence and photoacoustic intensity. Photoluminescence and photoacoustic intensity are typically inversely related due to competing radiative and non-radiative decay pathways when the light absorbance is constant.²⁵ However, we see here that both photoluminescence and photoacoustic signal increase with increasing size, which was very likely due to the increased light absorption by larger particles (**Figure 2.2a**).

We also studied the relationship between nanoparticle concentration and photoacoustic signal with the SiCNP620 because SiCNP620 showed the highest photoacoustic signals. The photoacoustic intensity of SiCNP620 was linearly dependent on the particle concentration (**Figure 2.3d**). The limit of detection of SiCNP620 was measured to be 10 $\mu\text{g/mL}$.

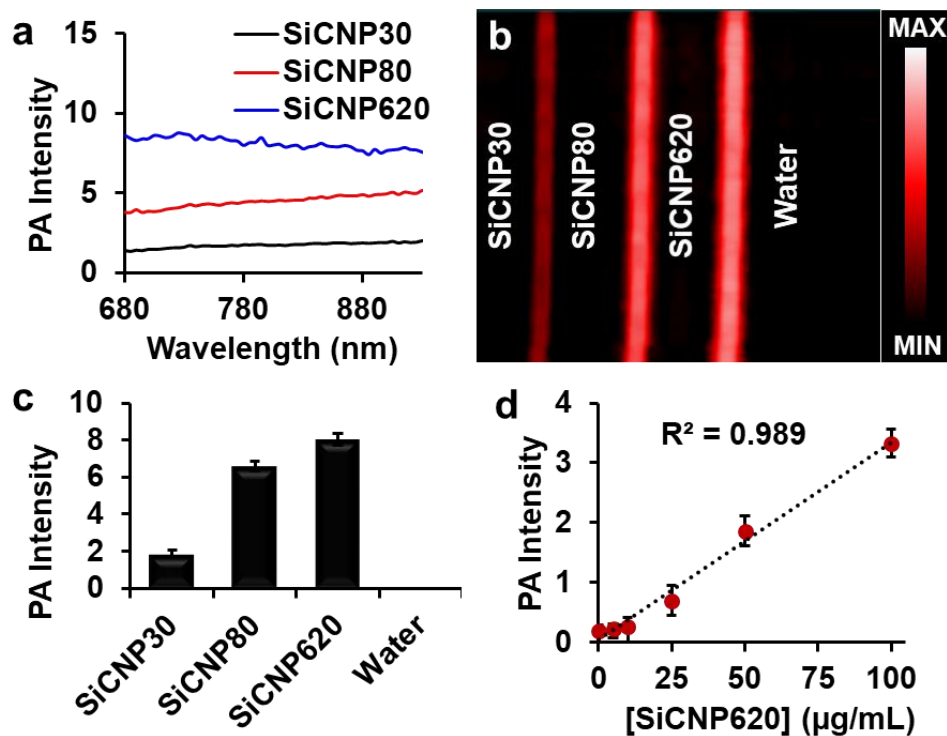


Figure 2.3. Photoacoustic properties of SiCNPs. (a) Photoacoustic spectra, (b) photoacoustic image and (c) corresponding quantification of photoacoustic intensity at 725 nm. (d) Photoacoustic signal of SiCNP620 was linearly dependent on the particle concentration. The limit of detection of SiCNP620 was found to be 10 $\mu\text{g/mL}$. Error bars are standard deviations of 5 regions of interests.

Given the strongest photoluminescence and photoacoustic signals of SiCNP620 among the three SiCNPs, we used SiCNP620 for stem cell imaging and tracking. We first studied the cytotoxicity of SiCNP620 to mouse mesenchymal stem cells at different concentrations *via* a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, or MTS, assay (Promega). No significant decrease in cell viability was found when the SiCNP620 concentration was smaller than 400 $\mu\text{g/mL}$ (**Figure 2.4a**). Moreover, the SiCNP620-labeled cells showed a similar growth rate compared to unlabeled cells at labeling concentrations of 50-200 $\mu\text{g/mL}$ (**Figure 2.4b**).

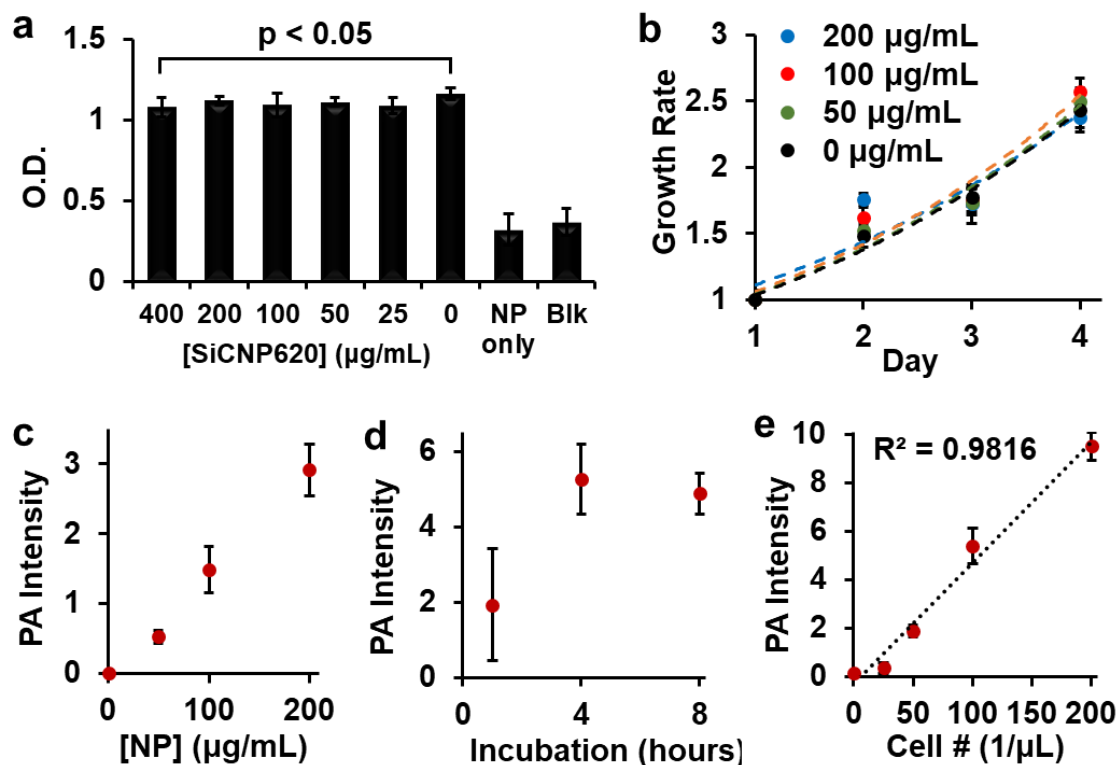


Figure 2.4. Biocompatibility and labeling conditions of SiCNP620 in mesenchymal stem cells. (a) No significant decrease in cell viability was seen for labeling concentrations smaller than 400 μg/mL. (b) Cells labeled with SiCNP620 at different concentrations showed similar growth rates to that of unlabeled cells. Error bars are standard deviations of 8 replicates. (c) A linear relationship is seen between labeling concentration and photoacoustic signal for four hours of labeling. (d) For a labeling concentration of 50 μg/mL, a labeling time of four hours was optimal. (e) Photoacoustic intensity was linearly dependent on the concentration of SiCNP620-labeled cells (50 μg/mL, 4 hours incubation), and the limit of detection was 37 cells/μL. Error bars are standard deviations of 5 measurements.

Then, we studied the labeling ability of SiCNP620 to mouse mesenchymal stem cells. The overall labeling capacity which is the average number of nanoparticles entered a cell was evaluated with photoacoustic imaging. The labeling concentration and incubation time affected the labeling capacity. The photoacoustic signal increases with increasing labeling concentration (**Figure 2.4c**), and a labeling time of 4 hours was found

to be optimal (**Figure 2.4d**). Cell labeling capacity was also quantified with thermogravimetric analysis. At labeling concentrations of 50 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$, the cell labeling capacity were 1.87 and 6.88 ng SiCNP/cell, occupying 0.25% and 0.93% of the cell volume, which was higher than reported for silica nanoparticles.²⁶ For all future experiments, stem cells were labeled with a particle concentration of 50 $\mu\text{g/mL}$ unless otherwise specified. There was a linear relationship between photoacoustic intensity and the number of SiCNP620-labeled cells (50 $\mu\text{g/mL}$) (**Figure 2.4e**), and the limit of detection of these labeled cells was approximately 37 cells/ μL .

The SiCNP620-labeled mesenchymal stem cells were visible under a fluorescence microscope using a Texas Red filter cube (the excitation is 585 nm and the emission is 624 nm). Unlabeled cells showed no photoluminescence signal (**Figure 2.5**). The photoluminescence of SiCNP620 was stable and the labeled stem cells showed strong photoluminescence signals even 11 days after labeling, suggesting the ability of SiCNP620 for long-term imaging and tracking of stem cells. Unlabeled and SiCNP620-labeled mesenchymal stem cells were treated with adipogenic and osteogenic induction media. The SiCNP620-labeling did not affect stem cell differentiation. Moreover, SiCNP620 could track mesenchymal stem cell-derived adipocytes and osteocytes (**Figure 2.5**). Interestingly, for the adipogenic induced cells, the SiCNP620 could enter and therefore track the lipid vesicles as well.

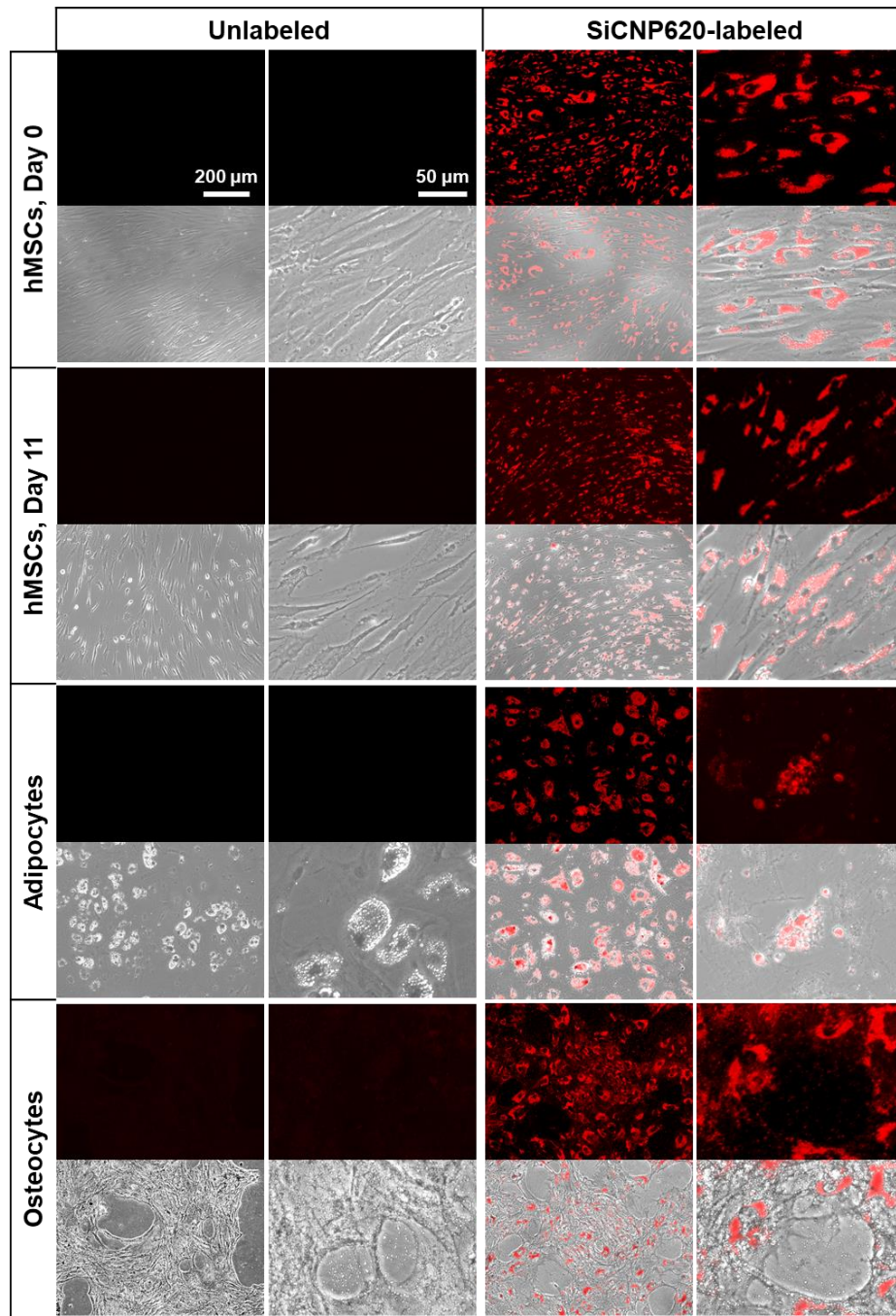


Figure 2.5. Fluorescence image (top) and its overlay with bright field image (bottom) of unlabeled and SiCNP620-labeled cells. SiCNP620-labeled mesenchymal stem cells are still visible under fluorescence microscope 11 days after labeling, indicating the long-term cell imaging ability of SiCNP620. In addition, adipocytes and osteocytes differentiated from SiCNP620-labeled mesenchymal stem cells still show photoluminescence three weeks after labeling.

To demonstrate the potential of these particles for *in vivo* cell tracking, we subcutaneously injected SiCNP620-labeled mouse mesenchymal stem cells into a nude mouse and scanned the cells with photoacoustic imaging. **Figure 2.6a** shows the photoacoustic spectra of injected cells. The photoacoustic intensity from 680 to 730 nm was decreased compared to the photoacoustic spectra of SiCNP620 only (**Figure 2.3a**), which may be due to the reduced tissue penetration of shorter wavelengths compared to the longer wavelengths. As a result, all cell implants were scanned in 3-dimensions at 770 nm. The photoacoustic intensity increased as the labeled cell concentration increased and then saturated when the cell concentration reached 20,000 cells/ μ L (**Figure 2.6b-c**). The *in vivo* limit of detection of SiCNP620-labeled cells was 7,800 cells/ μ L. Unlabeled cells showed no photoacoustic signal even at a high cell concentration. Continuous monitoring of the cell implants showed that we could still track SiCNP620-labeled cells for 14 days after injection via photoacoustic imaging (**Figure 2.6d**).

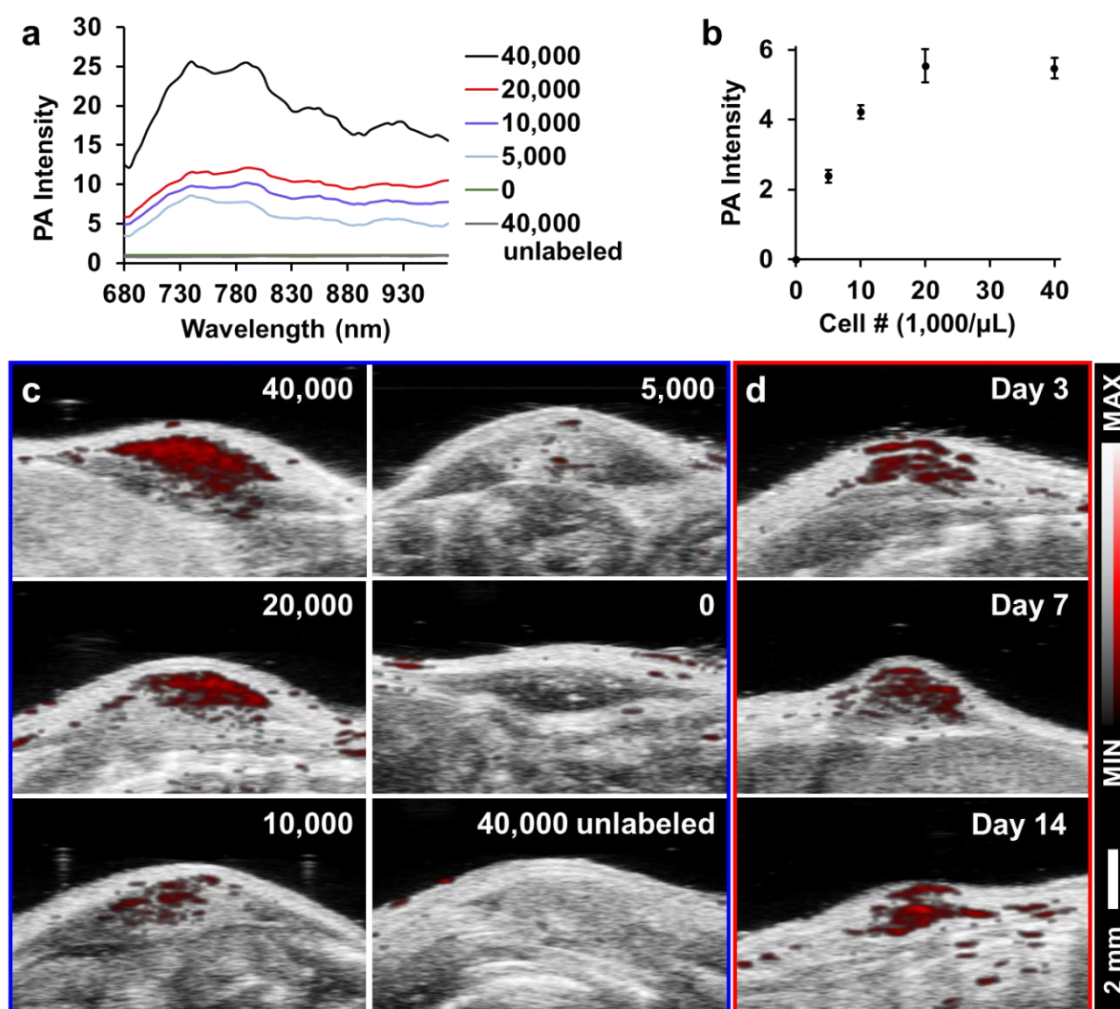


Figure 2.6. Tracking SiCNP620-labeled mesenchymal stem cells *in vivo*. (a) Photoacoustic spectrum of labeled MSCs implanted at varying cell concentrations. Labels represent injection concentrations in cells/μL. (b) Quantified photoacoustic intensity of injected SiCNP620-labeled cells at a wavelength of 770 nm. Error bars are standard deviations of 5 ROIs. (c) Photoacoustic and ultrasound overlay images of the injections. (d) Long-term tracking of the 20,000 cells/μL implant shows that cells are still visible over a period of 14 days.

2.4 Conclusion

Our future work involves understanding the mechanisms of photoacoustic excitation and optimizing the photoacoustic intensity of SiCNPs by surface modification. Coating with additional silica or etching could potentially change the photoacoustic intensity by reducing the thermal interfacial resistance between the nanoparticle and surrounding medium.²⁷ Ongoing work will be quantifying cells and monitoring the cell fate *in vivo* with photoacoustic imaging. The ability of these nanoparticles to track differentiated stem cells and other cells will also be performed in our next steps.

Long-term cell tracking is important to understand cell fate post-injection and provides insight for subsequent treatment. In this paper, we report for the first time that SiCNPs have intrinsic photoluminescent and photoacoustic signals, which enable SiCNPs to image cells *in vitro* and track cells *in vivo*. The SiCNPs were capable of long-term tracking of stem cells even after differentiation due to stable and strong luminescence. These nanoparticles showed excellent biocompatibility and labeling capacity for mesenchymal stem cells. Thus, SiCNPs are promising for long-term stem cell imaging and tracking.

2.5 Acknowledgements

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