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Folate Receptor-targeted Aggregation-enhanced Near-IR Emitting Silica Nanoprobe for One-photon *in vivo* and Two-photon *ex vivo* Fluorescence Bioimaging

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Abstract

A two-photon absorbing (2PA) and aggregation-enhanced near infrared (NIR) emitting pyran derivative, encapsulated in and stabilized by silica nanoparticles (SiNPs), is reported as a nanoprobe for two-photon fluorescence microscopy (2PFM) bioimaging that overcomes fluorescence quenching associated with high chromophore loading. The new SiNP probe exhibited aggregate-enhanced emission producing nearly twice as strong signal as the unaggregated dye, a three-fold increase in two-photon absorption relative to the DFP in solution, and approx. four-fold increase in photostability. The surface of the nanoparticles was functionalized with a folic acid (FA) derivative for folate-mediated delivery of the nanoprobe for 2PFM bioimaging. Surface modification of SiNPs with the FA derivative was supported by zeta potential variation and ¹H NMR spectral characterization of the SiNPs as a function of surface modification. *In vitro* studies using HeLa cells expressing folate receptor (FR) indicated specific cellular uptake of the functionalized nanoparticles. The nanoprobe was demonstrated for FRtargeted one-photon *in vivo* imaging of HeLa tumor xenograft in mice upon intravenous injection of the probe. The FR-targeting nanoprobe not only exhibited highly selective tumor targeting but also readily extravasated from tumor vessels, penetrated into the tumor parenchyma, and was internalized by the tumor cells. Two-photon fluorescence microscopy bioimaging provided three-dimensional (3D) cellular-level resolution imaging up to 350 μm deep in the HeLa tumor.

Keywords

Aggregation enhanced emission; near infrared emission; folate receptor targeting; two-photon absorption; two-photon fluorescence microscopy; silica nanoparticles; HeLa tumor; *in vivo* imaging; *ex vivo* imaging

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Supporting Information Available: Synthesis of DFP, preparation process and TEM image of DFP nanoaggregates, cell viability results, photostability and two-photon absorption cross section measurement methods are provided. ¹H NMR spectra of the SiNPs and compounds, as well as fluorescence images of organs showing nanoprobe distribution of mice injected with different SiNPs are also available. This material is available free of charge via the internet at <http://pubs.acs.org>.

Introduction

In comparison with conventional bioimaging techniques, such as computed tomography (CT) or magnetic resonance imaging (MRI), fluorescence-based optical bioimaging affords much higher sensitivity and has been widely exploited to track biological processes.^{1–4} Two-photon fluorescence microscopy (2PFM), one of the advanced fluorescence imaging techniques, has been investigated as a powerful tool for fundamental studies, cancer diagnosis, and oncologic drug development.^{5–9} This technique has a number of advantages over traditional one-photon fluorescence microscopy (1PFM), including highly localized 3D spatial excitation, lower photo-induced damage, longer possible observation time, less interference by autofluorescence, and deeper penetration in tissue and thick samples.^{10–13} A series of two-photon absorbing organic dyes¹⁴ was reported for 2PFM imaging while a number of nanoparticles such as gold nanorods¹⁵, quantum dots,¹⁶ and carbon nanoparticles¹⁷ also show promise in 2PFM applications. However, one major challenge to the development and implementation of 2PFM for molecular bioimaging is a lack of biocompatible probes with sufficient two-photon absorption (2PA) cross section, high fluorescence quantum yield, and high photostability in physiological environments.^{10, 18} For 2PFM applications, 2PA dyes are required to be hydrophilic or dispersible in aqueous media while maintaining high fluorescence efficiency.^{19–21} Generally, organic materials with large 2PA cross sections are synthetically more accessible in hydrophobic forms and their fluorescence efficiencies are dramatically reduced in aqueous media due to self-aggregation-induced fluorescence quenching.^{22, 23}

Recently, ceramic-based nanoparticles encapsulating hydrophobic dyes have been reported as biocompatible fluorescent probes for bioimaging.^{24–26} Although the strategy to disperse hydrophobic dyes in aqueous media has been relatively successful in improving photostability, the amount of dye remained at quite a low concentration, limiting the intensity of the fluorescence signal from individual nanoparticles due to aggregation-induced fluorescence quenching at high chromophore loading.²⁷ Typically, fluorescence quantum yields of organic dyes are decreased by self-quenching in the aggregated stage. However, a series of organic dyes with a self-distorted structure were reported to exhibit fluorescence enhancement via aggregation rather than the customary decrease.^{28–32} This phenomenon was exploited to develop a high-signal output silica nanoprobe for 2PFM imaging.³³ Although enhanced two-photon fluorescence was achieved, surface modification of the nanoparticles for *in vivo* targeting bioimaging have rarely been reported, and the 2PA cross section of the dyes employed were generally low. A series of compounds were reported to exhibit aggregation-enhanced emission,³⁴ but their two-photon based optical properties and application for 2PFM have not been reported. The development of silica nanoparticles (SiNPs) encapsulating an aggregation-enhanced emitting dye with a large 2PA cross section and high photostability as a nanoprobe for 2PFM would be significant.

To increase the stability of SiNPs for *in vivo* imaging, polyethylene glycol (PEG) is often introduced to their surface. PEGylation of a drug or therapeutic protein often "masks" the agent from the host's immune system, reducing antigenicity and immunogenicity. PEGylation also increases the hydrodynamic size of the agent, prolonging its circulatory time by reducing renal clearance. It also can decrease the toxicity of the system and provide water solubility to hydrophobic drugs and proteins.³⁵ The PEG group has also been used to reduce normal tissue uptake of various materials, decrease toxicity, and increase tumor accumulation.³⁶ Beyond passive targeting through the enhanced permeability and retention (EPR) effect, delivery of particles to smaller solid tumors and metastatic cells can be achieved by modifying particle surfaces with moieties directed at cell surface markers unique to the tumor cells.³⁷ With developments in cell biology, a variety of disease-specific ligand-receptor pairs have been identified, e.g., ligands based on antibodies, antibody

fragments, proteins, and peptides.³⁸ Because of their high selectivity to specific cell receptors, ligands based on antibodies, antibody fragments, proteins, and peptides were fervently investigated for drug and bioimaging agent delivery.³⁹ Among the receptors of interest, folate receptor (FR) is overexpressed by a spectrum of malignant tumors, including cancers of lung, ovary, breast, brain, kidney, colon and endometrium.⁴⁰ Recently, folic acid conjugated nanoparticles were investigated as carriers for delivering magnetic agents for MRI bioimaging,⁴¹ photosensitizers for photodynamic therapy,⁴² anti-cancer drugs for cancer therapy,⁴³ and quantum dots for two-photon fluorescence microscopy.⁴⁴ Folic acid has been widely used for selective delivery of attached imaging and therapeutic agents to tumors because of its high affinity for the folate receptor ($K_d = 10^{-10}$ M).⁴⁵ However, the cellular-level understanding of how nanoparticles distribute within a solid tumor has not been thoroughly investigated due to the lack of fluorophores suitable for high resolution 3-D analysis in deep tissues. Herein, a two-photon absorbing, aggregation-enhanced near infrared (NIR) emitting and folate receptor-targeting silica nanoprobe is reported to probe the cellular distribution of folate nanoprobe within a solid tumor. The FR-targeted, highly selective tumor accumulation of the new SiNP probe was demonstrated by *in vivo* one-photon fluorescence imaging of tumor-bearing mice and *ex vivo* two-photon fluorescence imaging of whole-mounted tumors. Our study presents a critical advancement in SiNP technology for disease diagnostics and therapeutics.

Experimental section

Materials and methods

Triethoxyvinylsilane (VTES, 97%), 1-butanol (99%), folic acid (FA), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 2-aminoethanethiol, and the surfactant Tween-80, N'-[3-(trimethoxysilyl)propyl]diethylenetriamine (DETA), were obtained from Sigma-Aldrich. N-Methyl-2-pyrrolidinone (NMP, 99%), triethylamine (TEA), and dimethyl sulfoxide (DMSO) were obtained from Acros. 2-(2,6-Bis((E)-2-(7-(diphenylamino)-9,9-diethyl-9H-fluoren-2-yl)vinyl)-4H-pyran-4-ylidene)malononitrile (DFP) was prepared as described in the Supporting Information. Maleimide-poly(ethylene glycol)-succinimidyl carboxymethyl, average M.W. 3400 (MAL-PEG-SCM) was obtained from Laysan Bio Inc. Rat anti-mouse CD31 was obtained from BD Biosciences. Alexa Fluor[®] 350 goat anti-mouse IgM was obtained from Invitrogen. The preceding chemicals or biological reagents were used as received without further purification, except where otherwise noted. Thermo Slide-A-Lyzer[®] 10K cut off dialysis cassettes and 0.22 μ m cutoff membrane filters were obtained from Fisher Inc. HeLa and MG63 cells were obtained from ATCC (America Type Culture Collection, Manassas, VA, USA). All cells were incubated in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA), 100 units/mL penicillin-streptomycin (Atlanta Biologicals, Lawrenceville, GA, USA), and incubated at 37 °C in a 95% humidified atmosphere containing 5% CO₂. Details of pyran derivative DFP, including preparation and characterization, will be reported elsewhere. ¹H NMR spectra were recorded at 300 MHz.

Synthesis of dye-encapsulated amine terminated SiNPs

The amine and PEG terminated nanoparticles, with or without encapsulating the fluorenyl derivative DFP, were synthesized according to the method described by Prasad *et al.* with moderate modification.²⁸ Briefly, the nanoparticles were prepared by coprecipitating the dyes with polymeric organically modified silica sol in the nonpolar core of Tween-80/1-butanol micelles in deionized water. NMP was used as a hydrophilic solvent, which has unlimited water miscibility as well as suitable solubility for DFP. First, 1.0 g of VTES in 10 mL NMP was hydrolyzed and condensed in the presence of 200 μ L ammonium hydroxide

(28.0–30%) at room temperature for 16 h to obtain a clear solution of prepolymerized silica sol. Then, the prepolymerized silica sol was filtered with a membrane filter (0.22 μm pore size) for further SiNP preparation. Micelles were prepared with 0.2 g of Tween-80 and 0.4 mL of 1-butanol in 10 mL of deionized water. To obtain dye-encapsulation in SiNPs, 100 μL of the sol solution was homogeneously mixed with 0.38 mL of NMP solutions without or with DFP (0 mg, 0 wt %; 0.9 mg, 10 wt %; 1.8 mg, 20 wt %; 2.7 mg, 30 wt %), followed by addition of 0.4 mL of the NMP solution by one-shot syringe injection into the prepared micelle dispersions to induce nanoprecipitation under vigorous magnetic stirring. After 30 min, 20 μL EDTA was added to functionalize the surface of the nanoparticles with amine. To ensure completion of sol-gel condensation within the coprecipitated nanoparticles, the mixtures were further stirred at room temperature for 24 h. Nanoparticle purification was conducted by dialyzing the dispersion against deionized water in a 10 kDa cutoff cellulose membrane to remove Tween-80 and 1-butanol for 48 h. The dialyzed solution was then filtered through a 0.22 μm cutoff membrane filter. The resulting nanoparticles were stored at 4 $^{\circ}\text{C}$ for later experiments.

Conjugation of amine-terminated SiNPs with MAL-PEG-SCM

To 10 mL of DFP-doped silica nanoparticles dispersed in deionized water, 0.5 mL PBS (20 $\times$, pH = 7.4) and 10 mg MAL-PEG-SCM (M. W. 3400) in 300 μL water were added. The mixture was stirred for 30 min in the dark. The unreacted MAL-PEG-SCM polymer was removed by dialyzing the dispersion against deionized water in a 10 kDa cutoff cellulose membrane for 48 h. Finally, the dialyzed solution was filtered through a 0.22 μm cutoff membrane filter and stored at 4 $^{\circ}\text{C}$ for later experiments.

Surface modification of maleimide-terminated SiNPs with FA

To 2 mL of the MAL terminated SiNPs dispersed in deionized water, 0.1 mL PBS (20 $\times$, pH = 7.4) and (S)-2-(2-(4-((2-amino-4-hydroxypteridin-6-yl)methylamino)phenyl)acetamido)-5-(2-mercaptoethylamino)-5-oxopentanoic acid (FA-SH) (1 mg/mL in DMSO, 500 μL) was added, and the mixture was stirred for 1 h. The unreacted FA-SH and DMSO were removed by dialyzing the reaction mixture against deionized water in a 10 kDa cutoff cellulose membrane for 48 h. Finally, the dialyzed solution was filtered through a 0.22 μm cutoff membrane filter and stored at 4 $^{\circ}\text{C}$ for subsequent experiments.

Characterization of dye-encapsulating SiNPs

Transmission electron microscopy (TEM) images were obtained using a JEOL-JEM 1011 transmission electron microscope (JEOL, Ltd., Japan.) operating at 100 kV in bright field for the nanoparticles. A drop of SiNPs dispersed in water was placed on a holey carbon film copper grid and left to evaporate. The zeta potential measurement and particle size analysis by a light scattering method were conducted using the Zetasizer Nano-ZS90 (Malvern Instruments). ^1H NMR spectra of SiNPs dispersed in D_2O were recorded at 300 MHz.

Uptake of SiNPs by cancer cells

HeLa or MG63 cells were placed onto poly-D-lysine coated glasses in 24-well plates (15,000 cells per well), and the cells were incubated for 48 h before incubating with dye-doped SiNPs. Stock solution of dye-doped SiNPs suspended in water was prepared as ~50 μM solution. The solution was diluted to 0.1, 1, and 5 μM solutions by complete growth medium, RPMI-1640, and freshly placed over the cells for a 2 h period. After incubation, the cells were washed with PBS for 3 min at 37 $^{\circ}\text{C}$ (3–5 $\times$) and fixed using 3.7% formaldehyde solution for 15 min at 37 $^{\circ}\text{C}$. To reduce autofluorescence, a fresh solution of NaBH_4 (1 mg / mL) in PBS (pH = 8.0), which was prepared by adding few drops of 6N NaOH solution into PBS (pH = 7.2), was used for treating the fixed cells for 15 min (2 $\times$). The plates were then

washed twice with PBS and once with water. After this, the glass coverslips were mounted by Prolong gold mounting medium for microscopy.

Animal model, in vivo and ex vivo imaging

Eight-week-old female athymic nude mice were obtained from Harlan Laboratories (Indianapolis, IN). HeLa cells (5×10^6 cells) were inoculated subcutaneously into the right thighs of mice. Imaging studies were performed 10 days after the tumor inoculation; SiNPs dispersed in PBS (300 μ M, ~ 0.2 mL, 3 nmol/g body weight) or vehicle (PBS, ~ 0.2 mL) were injected to the mice via the tail vein. At different times after the injection of SiNPs or vehicle, the mice were imaged using an IVIS Spectrum imaging system (Caliper Life Sciences) while under anesthesia. Fluorescence images were recorded by two channels. One channel, excitation at 500 nm, and emission at 720 nm, was used to record the fluorescence from the SiNPs, while the other channel, excitation at 605 nm and emission at 720 nm, was used to record background tissue autofluorescence. The resulting images were processed by subtracting the background tissue autofluorescence from the fluorescence from the SiNPs with software of the IVIS Spectrum imaging system. After *in vivo* imaging, the mice were euthanized with i.v. injections of xylazine and the tissue samples were collected after 20 mL PBS perfusion through the heart, fixed in 4% paraformaldehyde, and cryoprotected in PBS solution for *ex vivo* one- and two-photon fluorescence microscopy imaging. Two mice were examined for each sample.

1PFM cell, tissue and ex vivo 2PFM imaging

Conventional single-photon fluorescence cell images were obtained using an inverted microscope (Olympus IX70) equipped with a QImaging cooled CCD (Model Retiga EXi) and excitation with the filtered emission of a 100 W mercury lamp. In order to improve the fluorescence background-to-image ratios, one-photon confocal fluorescence images of the fixed cells were taken using a custom made filter cube for dye-doped SiNPs. The specifications of the filter cube were tailored to match the excitation wavelength of probes and to capture most of its emission profile. 1PFM imaging of *ex vivo* tissue sections was performed on a Leica TCS SP5 II Tandem Scanner upright multiphoton laser scanning microscopy system. Frozen tumors were sectioned by a freeze microtome (Leica, CM1900) to ~ 20 μ m thick sections. For labeling the endothelia of blood vessels, MEC13.3 antibody (1:50 dilution in PBS) was used to react with CD31, and a labeled goat anti-mouse IgM antibody Alexa Fluor[®] 350 (1 μ g/mL in PBS) was used to label the primary antibody MEC13.3. The nuclei of the sectioned tissues were stained with Hoechst 33285 (0.2 μ g/mL in PBS). The section tissues were imaged and examined by laser-scanning confocal microscopy (Leica, TCS SP5). 2PFM cell imaging was performed using a modified Olympus Fluoview FV300 microscope system coupled to a tunable Coherent Mira 900F Ti: sapphire laser (76 MHz, modelocked, 200 femtosecond pulse width, tuned to 840 nm). A short-pass emission filter (cutoff 690 nm) was placed in the microscope scanhead to avoid background irradiance from the excitation source. Consecutive layers, separated by approximately 0.15 μ m for cell imaging and 0.3 μ m for tumor imaging, were recorded to create a 3D reconstruction from overlaid 2PFM images. Two-photon induced fluorescence was collected with a 60 $\times$ microscope objective (UPLANSAPO 60 $\times$, NA = 1.35, Olympus). 2PFM thick tissue imaging was performed using a Leica TCS SP5 microscope system coupled to a tunable Coherent Chameleon Vision S (80 MHz, modelocked, 75 femtosecond pulse width, tuned to 980 nm). A short-pass emission filter (cutoff 840 nm) was placed before the CCD camera to avoid background irradiance from the excitation source. Consecutive layers, separated by approximately 0.5 μ m for thick tumor imaging, were recorded to create a 3D reconstruction from overlaid 2PFM images. Two-photon induced fluorescence was collected with a water immersion high NA/low magnification objective

lens HC X APO L20× (NA = 1.0, Leica) with the magnification changer in the CCD camera mode (3× magnification was used).

Results and discussion

Aggregation enhanced emission of DFP

To study and exploit the potential utility of pyran derivatives, which were reported to exhibit aggregation-enhanced emission,³⁰ a pyran derivative 2-(2,6-bis((E)-2-(7-(diphenylamino)-9,9-diethyl-9H-fluoren-2-yl)vinyl)-4H-pyran-4-ylidene)malononitrile (DFP) (Figure 1, left) was prepared and its photophysical properties were investigated herein for 2PFM bioimaging. A simple precipitation method was employed to prepare the stable DFP nanoaggregates dispersed in water,^{24, 26} with THF as a water-miscible solvent for DFP. The roles of water in the organic nanoparticle preparation by precipitation included lowering the solubility of DFP and stabilizing a colloidal dispersion by promoting surface charge. Figure S1 shows a representative transmission electron microscopy (TEM) image of the nanoaggregates made from 90% v/v of water in THF, having a diameter of 20 ± 6 nm. The results pictured in Figure 1 (right) indicated that the relative fluorescence quantum yield (Φ_f) of DFP with the same concentration of 5×10^{-6} mol·L⁻¹ decreased dramatically from 0.067 to 0.003 when the volume fraction of the water increased from 0 to 40%. Bulk precipitation occurred in the range of 50–60% water volume fraction, possibly because this amount of water decreased the solubility of DFP but did not stabilize the colloid enough by promoting surface charge.²⁴ The relative fluorescence quantum yield, Φ_f , of DFP at 50–60% was not measured due to bulk precipitation. After the volume fraction of the water was increased above 60%, stable nanoaggregates of DFP formed, and the relative Φ_f of DFP under the same conditions varied from 0.09 to 0.15 with the volume fraction of water increasing from 70 to 90%. It is apparent that the fluorescence of DFP was intensified by aggregation. Figure 1(middle) illustrates that the relative fluorescence intensity of DFP with the same concentration at the aggregate state was much stronger than it was in THF solution under 500 nm laser excitation. The absorption and emission spectra of DFP in different water fraction solutions are presented in Figure S2. The emission spectra showed that the fluorescence intensity was enhanced by aggregation with a minimal shift, whereas the absorption of DFP broadened and the intensity decreased with increasing water.

This relatively abnormal phenomenon exhibited by DFP may be explained by rotation upon the axes of the olefinic double bonds adjacent to the fluorenyl rings and pyran ring in DFP (Figure 1, left) so the conformational flexibility may result in the molecule being relatively nonemissive in dilute solution. However, when the molecule formed nanoaggregates in more hydrophilic solution, the intramolecular rotations were restricted and the molecular conformation was stiffened, resulting in aggregation-enhanced emission (i.e., the favored conformations were radiative).^{34, 46} This presumption was supported by the blue-shifted monomer fluorescence in a dilute solid solution of DFP in a polymethylmethacrylate (PMMA) film, where isolated molecules are stiffened and distorted by the rigid matrix (Figure 4a). The DFP emission is red-shifted without quenching at concentrations that normally would be high enough to induce self-quenching, because the intermolecular steric hindrance between DFP molecules not only extends the π -conjugation but also diminishes intermolecular quenching effects.⁴⁷

Synthesis, characterization and photophysical properties of amine-terminated DFP doped SiNPs

To apply the observed aggregation-enhanced emission property of the hydrophobic 2PA dye DFP for 2PFM bioimaging, dye-doped SiNPs encapsulating different amounts of DFP were prepared according to a moderately modified reported method.³³ Dye-doped SiNPs have

been reported to be biocompatible,^{48, 49} stable without releasing encapsulated hydrophobic molecules,⁵⁰ and facile to be modified with biomolecules.⁵¹ The synthesis and surface modification of the dye-doped SiNPs are schematically described in Scheme 1. To prepare the high loading dye-doped or nonlabeled SiNPs, a prepolymerized triethoxyvinylsilane (VTES) sol solution was first prepared. Then the sol solution and a solution with or without a certain amount of DFP were mixed and co-precipitated within the nonpolar interior of aqueous Tween-80 micelles through a solvent displacement process.⁵² N'-[3-(Trimethoxysilyl)propyl]diethylenetriamine (DETA) was added to the reaction mixture to introduce free amine groups on the SiNP surface for subsequent bioconjugation.

The zeta potential was analyzed to determine the change in surface charge of the SiNPs. Results in Figure 2a indicated that the nonlabeled SiNPs presented an overall positive charge with a zeta potential value of $+10.4 \pm 0.5$ mV, presumably because of the presence of amine moieties on the surface of the SiNPs.⁵² With increasing dye loading, the overall surface charge of the SiNPs became more and more negative, and the 30 wt% (defined as DFP/[DFP + VTES] by weight) loading SiNPs exhibited a zeta potential of $+2.14 \pm 0.4$ mV. The overall surface charge of SiNPs encapsulating different amounts of DFP varied possibly due to increasing surface hydrophobicity that promoted preferential adsorption of anions (OH^-) on the surface. The SiNP particle size was analyzed by transmission electron microscopic (TEM) and dynamic light scattering (DLS). The results from DLS (Figure 2b) indicated the particle size of SiNPs increased with increased loading ratios of DFP in the particles. A representative TEM image of 20 wt% SiNPs (Figure 2c) was in good agreement with their number-averaged size distribution in water. The diameters were in the range of 20–30 nm with an average diameter of ca. 25 nm. This size is small enough to minimize the clearance of the host's immune system, prolong the circulation time of the SiNPs in live animals, and, hence, increase accumulation of the SiNPs in the targeted tumor.

The fluorescence quantum yield, Φ_f , and luminescence lifetime, τ_f , of the dye-doped SiNPs encapsulating various amounts of DFP, dispersed in water, were analyzed to optimize conditions for their use in 2PFM bioimaging. The results shown in Figure 3a indicate that the fluorescence quantum yield, Φ_f , achieved a highest value of 0.19 ± 0.02 at a 20 wt% DFP concentration. According to the fluorescence decay presented in Figure 3b, the fluorescence lifetime of the SiNPs reached the highest value (2.32 ± 0.08 ns) at 20 wt% DFP and lowest value (1.81 ± 0.08 ns) at 10 wt% DFP, consistent with fluorescence quantum yield measurements.

The 20 wt% SiNPs were chosen for the 2PFM bioimaging, not only because of their higher fluorescence quantum yield but also due to their suitable particle size for *in vivo* bioimaging. The linear and nonlinear photophysical properties of silica nanoprobe were investigated in phosphate buffered saline (PBS, 1×) to determine the potential utility of the silica nanoprobe for 2PFM bioimaging applications. The emission spectra of the 20 wt% SiNPs matrix was close to the emission spectra of 20 wt% DFP in PMMA (Figure 4a), indicating that DFP in 20 wt% SiNPs was in the same aggregate state as DFP in PMMA solid solution. The absorption, emission, excitation, 2PA cross section, as well as the excitation anisotropy spectra of the silica nanoprobe in PBS (1×) are shown in Figure 4b. The linear absorption spectrum (dark) and excitation spectrum (green) were sufficiently close to each other, and the emission spectrum (red) showed a maximum emission in the red (~650 nm), extending into the near-IR. The values of excitation anisotropy (Figure 4b, blue) were not constant in the range of the main 1PA band (450 – 550 nm), indicating its relatively complex nature. Presumably, this band corresponds to at least two electronic transitions with different orientations of its transition dipoles.

By employing a standard z-scan method⁵³ with a femtosecond laser system, the silica nanoprobe afforded an impressive maximum 2PA cross section (δ) of ~ 2100 GM ($1 \text{ GM} = 10^{-50} \cdot \text{cm}^4 \cdot \text{s} \cdot \text{photon}^{-1}$) at 980 nm (Figure 4b, triangle), which was ca. three times higher than free DFP in THF (Figure 4b, star). The aggregation enhanced two-photon absorption of DFP in SiNPs may be due to the considerably hindered internal rotation of the dye molecule in the solid silica matrix.²⁸ The 2PA efficiency of our nanoprobe is much higher than those previously reported, typically < 300 GM, for two-photon fluorescence microscopy (2PFM) bioimaging.⁵⁴

Surface modification, characterization and photophysical properties of the FA conjugated DFP doped SiNPs

In order to mask the silica nanoprobe from the host's immune system and target FR overexpressing tumors, the surface of the SiNPs was further modified with maleimide-terminated PEG and then FA. The succinimidyl carboxymethyl (MAL-PEG-SCM, M.W. 3400) was first used to react with the amine group on the surface of the SiNPs, yielding a maleimide functional group on the surface to react with the thiol group on (S)-2-(4-((2-amino-4-hydroxypteridin-6-yl)methylamino)phenyl) acetamido)-5-(2-mercaptoethylamino)-5-oxopentanoic acid (FA-SH) in the next step (Scheme 1). Finally, the FA-conjugated SiNPs were purified by dialysis to remove unreacted molecules. The success of FA conjugation to the SiNPs was confirmed by zeta potential measurement (Figure 5a). The zeta potential value of amine-terminated SiNPs (SNP-DFP-NH₂) decreased from +2.8 mV to -6.4 mV after reaction of surface amino groups with MAL-PEG-SCM to form the MAL-terminated SiNPs (SNP-DFP-PEGMAL) since most of the positive charge from NH₃⁺ was eliminated. After FA-SH conjugation with SNP-DFP-PEGMAL and formation of FA-terminated SiNPs (SNP-DFP-PEGFA) the zeta potential value decrease continuously to -13.8 mV, possibly because the positive charge on the surface was shielded by the FA derivative.

In addition, proton NMR spectra of the SiNPs in D₂O, as well as SCM-PEG-MAL and Tween-80 in D₂O, FA-SH in DMSO-d₆, DFP in CD₃Cl (Figure 5c and ¹H NMR spectra in Supporting Information), confirmed the surface modification of the DFP-doped SiNPs modified by FA. A signal at $\delta = 6.76$ ppm from the maleimide was present after the amine terminated SiNPs reacted with MAL-PEG-SCM, producing maleimide groups on the surface of the SiNPs. Then the signal from maleimide at $\delta = 6.76$ ppm disappeared after the maleimide reacted with the thiol of FA-SH, and the signal at $\delta = 7.62$ ppm from folic acid appeared in the proton NMR spectrum of FA-modified SiNPs. Due to the presence of PEG on the surface of the SiNPs, the FA-conjugated SiNPs dispersed well in phosphate buffered saline (PBS, 1×) solution and was quite stable. A representative TEM image and dynamic light scattering (DLS) size distribution of the FA conjugated nanoparticles dispersed in PBS is shown in Figure S5. The average particle size was ~ 28 nm with a polydispersity index (PdI) value of 0.375 and no aggregation or decomposition was observed through all experiments.

The photostability of SNP-DFP-PEGFA in PBS was evaluated resulting in a photodecomposition quantum yield, Φ_d , of 3.7×10^{-7} that was ca. 25% of the value for DFP in Tween-80 micelles in PBS (1.3×10^{-6}). This result is significant in that the photostability of the DFP probe encased in the SiNP (SNP-DFP-PEGFA) was ca. four times higher than free DFP (in solution). It is possible that when the nanoprobe was irradiated, the silica matrix protected the dye molecules from the aqueous environment and reduced the oxidation of the dye molecules by slowing the diffusion of oxygen, an aspect of future investigation.

The linear and nonlinear photophysical and photochemical properties of the silica nanoprobe resulted in a highly desirable figure of merit ($F_M = \delta\Phi_f/\Phi_d$) value of $\sim 1.1 \times 10^9$ GM in

PBS, a value that is about two times higher than a previously reported high-fidelity small molecule probe.⁵ The absorption and emission spectra of SNP-DFP-NH₂, SNP-DFP-PEGMAL, and SNP-DFP-PEGFA are presented in Figure 5b, and after FA conjugation the absorption of FA in the short wavelength was presented, and, as can be seen, the emission spectrum did not change.

Cytotoxicity, in vitro 1PFM and 2PFM folate receptor-targeted cell imaging of the DFP SiNP nanoprobe

Before applying the FA-conjugated SiNPs for FR-targeted bioimaging, the probe's cytotoxicity with HeLa and MG63 cells was investigated using an MTS assay.⁵ The results (Figure S4) indicated that the FA-conjugated SiNPs are compatible with these cell lines (~85% cell viability) over a DFP concentration range from 0.01 – 5 μ M, which was high enough for imaging. DFP concentrations were determined from the absorption of SNP-DFP-PEGFA at 472 nm (absorbance coefficient, $\epsilon_{472\text{nm}} = 5.4 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in PBS). The results suggested that the FA-conjugated SiNPs will be suitable for FR-targeted 2PFM *in vitro* cell imaging.

In preparation for *in vivo* imaging studies, *in vitro* cell imaging was conducted to test the selectivity of the FA-conjugated silica nanoprobe to FRs. HeLa cells, known to overexpress FRs, and low FR expressing MG63 cancer cells were incubated with the same concentration of the FA-conjugated SiNPs for the same time. 1PFM and 2PFM images are presented in Figure 6 demonstrating that HeLa cells (top row) incubated with FA-conjugated SiNPs (SNP-DFP-PEGFA) were much brighter than the MG 63 cells (a negative control, second row from the top) incubated with the same nanoprobe. In addition, two additional control experiments were performed. In one control, HeLa cells (third row from the top) incubated with non-FA-conjugated SiNPs (SNP-DFP-PEGMAL) under the same conditions showed no significant uptake of the probe. In the additional control experiment HeLa cells (bottom row) whose FRs were first blocked with FA and, subsequently incubated with FA-conjugated SiNPs, also demonstrated no specific uptake of the SiNPs. These results indicated that the HeLa cancer cells, which are well known to overexpress FRs, internalized the FA-conjugated SiNPs, presumably by FR mediation, but those cells which do not overexpress FRs or whose FRs were blocked did not uptake the silica nanoprobe effectively.

Folate receptor-targeted in vivo tumor imaging with nanoprobe and biodistribution study

The *in vitro* studies demonstrated that the FA-conjugated SiNPs can target FRs, providing motivation to investigate the SiNPs for FR-targeting bioimaging in mice. Athymic nude mice bearing HeLa tumors (10 days post inoculation of 5×10^6 HeLa cells on the right thigh), which overexpress FRs, were administered with SNP-DFP-PEGFA or SNP-DFP-PEGMAL probe (3 nmol DFP per gram of animal body weight) via tail vein injection. The mice were imaged at different time points post injection (p.i.), using an IVIS Spectrum imaging system; the fluorescence signals from the nanoprobe were collected in one channel with excitation at 500 nm and emission at 720 nm. The tissue background autofluorescence signals were recorded with a second channel with excitation at 605 nm and emission at 720 nm. The efficiency of the channels to separate the probe fluorescence signals from the background autofluorescence signals was checked by imaging standard samples with different concentrations of SNP-DFP-NH₂ in PBS (Figure S6). In this experiment, two groups of mice were analyzed in the same way. The resulting images presented in Figure 7 were corrected by subtracting the animal autofluorescence background images from the silica probe fluorescence images using the vendor software. Just after 30 min p.i., a fluorescence signal was detected in the tumor. A steady increase in the tumor fluorescence intensity in the mice injected with SNP-DFP-PEGFA was observed during the next few hours and the tumor fluorescence intensity reached its maximum at 6 h p.i. No significant

fluorescence signal was detected in the tumor for the SNP-DFP-PEGMAL injected mouse. These results indicate that the FA-conjugated SiNPs accumulated in the tumor, and demonstrated that the FA-conjugated nanoprobes can be delivered to tumor sites effectively.

At 6 h p.i., the mice injected with SNP-DFP-PEGFA or SNP-DFP-PEFMAL were sacrificed, and their HeLa tumors and organs were harvested and imaged immediately with the IVIS spectrum imaging system using the same collection channels described above. The fluorescence images of the organs and tumors are shown in Figure S7. The SiNPs' biodistribution was determined by their relative fluorescence intensity from these organs (Figure 8a). The biodistribution of the nanoprobes in normal organs, e.g. liver, kidney, spleen, heart, lung, intestine, muscle, and brain, were similar between the FA-conjugated and non-conjugated probes and consistent with the biodistribution of other SiNPs reported previously.²⁶ The most important difference in the biodistribution was the ca. three times higher HeLa tumor uptake observed for FA-conjugated SiNPs, supporting FR-mediated accumulation of the nanoparticles. For a better comparison and higher resolution, the excised tumors of the mice injected with SNP-DFP-PEGFA or SNP-DFP-PEGMAL were imaged and presented in Figure 8b. The fluorescence signal intensity of the tumor from the mouse injected with SNP-DFP-PEGFA was ca. three times higher than the one from the mouse injected with SNP-DFP-PEGMAL. The efficiency of tumor targeting of the FA-conjugated nanoprobe was impressive, particularly considering that the mice used in this study were fed a regular diet rather than a folate-free diet, which is usually necessary to increase the detection sensitivity of the folate receptor-targeted probes.⁵⁵ In addition, because the *ex vivo* tumor fluorescence signal appeared to be fairly homogenous and FRs are overexpressed by cancer cells, it is reasonable to predict that the fluorescence signal should emanate from cells distributed throughout the tumor rather than being localized in the vasculature, an aspect explored in the following section.

Histology studies of HeLa tumors from the mice administered with nanoprobes

After *in vivo* and *ex vivo* imaging of the tumor by the IVIS spectrum imaging system, the collected tumors were sectioned to ~20 μm thick sections. In order to determine the location of the nanoprobe in the tumor, the nuclei of the tumor were stained with Hoechst 33285 before visualizing with a Leica 1P-FCS confocal microscope. The confocal fluorescence image of the FA-conjugated nanoprobe accumulated in the tumor together with the localized fluorescence spectrum is illustrated in Figure 9. The 1PFM image of the tissue indicated that the FA-conjugated nanoprobe accumulated in the tumor cells (Figures 9d and 9f). The HeLa cancer cells internalized the nanoprobe and accumulated in specific organelles within the cancer cells (likely endosomes), and the localized fluorescence spectrum (Figure 9e) confirmed that the localized fluorescence (pointed out by triangles in Figure 9f) was from the internalized nanoprobe ($\lambda_{\text{em max}} = 650 \text{ nm}$) rather than tissue autofluorescence ($\lambda_{\text{em max}} = 524 \text{ nm}$), and the fluorescence signals from the folate nanoprobe was about three times higher than the autofluorescence signals.

Significantly, no fluorescence for the nanoprobe channel was observed in the HeLa tumors from the mice injected with non-folic acid-conjugated nanoprobe SNP-DFP-PEGMAL (Figure S8). In addition, blood vessels were stained to show how far the nanoprobe penetrates into the tumor; the result shown in Figure S9 indicated that the folate nanoprobe leaked out of the blood vessels (Figure S9, pointed out by triangles) and spread throughout the tumor.

2PFM folate receptor-targeted imaging of HeLa tumors

The tumors excised from the nanoprobe-administered mice were whole-mounted and imaged, optical layer-by-layer, using a 2PFM imaging system. The fs laser excitation was

tuned to 980 nm in order to achieve cellular-level 2PFM images of the tumors. The thick 2PFM images of a tumor are presented in Figure 10. The 2PFM tumor images showed that the FA-conjugated nanoprobe appeared to be primarily distributed in the cytoplasm of cancer cells (a few cells are indicated by arrowheads in Figure 10c) though some may be associated with the cell membrane. A deep tissue 2PFM imaging (ca. 350 μm) was achieved (Figure 10e) with cellular-level visual information of a solid tumor. While the tumor from the mouse that was administered with FA-conjugated SiNPs clearly showed cells with SiNP accumulation (Figure 10a), no significant signal was observed for non-FA-conjugated SiNPs (Figure S10). The results are consistent with FR-binding of the FA-conjugated SiNPs and accumulation in the tumor cells. Thus, imaging nanoprobe can be effectively and specifically delivered to tumors that overexpress FRs. These results suggest that the FA-mediated active targeting strategy is more effective than passive targeting strategies via enhanced permeability and retention (EPR).

Conclusion

In this study, we have developed an aggregation-enhanced long wavelength emission, 2PA pyran derivative (DFP) that exhibits much stronger fluorescence upon aggregation than in THF solution. In order to harness the potential of the hydrophobic DFP probe for biological imaging and stabilize its aggregation, DFP-encapsulating SiNPs that target folate receptors were synthesized and characterized. In order to specifically deliver the two-photon fluorescent SiNPs to tumor, the surface of the nanoparticles was functionalized with PEG and then modified with a folic acid derivative at the outer terminus of the PEG group. Nanoparticle surface functionalization was characterized by measuring changes in the zeta potential and NMR spectra of the SiNPs. The DFP aggregate-encapsulating SiNPs exhibited enhanced fluorescence emission (twice the fluorescence quantum yield relative to unaggregated DFP). Importantly, these nanoparticles exhibited a three-fold increase in two-photon absorption relative to the DFP in solution. In addition, the photostability of the fluorescent SiNPs was ca. four times higher than the free DFP dye, resulting in an impressively high figure of merit for the new nanoparticle probe. TEM and DLS provided information regarding nanoparticle size and size distribution, indicating an average particle size of ca. 25 nm.

The cytotoxicity of the new SiNPs was assessed in two cell lines and found to be suitable for *in vitro* cell imaging. The FA-modified DFP-containing SiNPs were selectively uptaken by HeLa cells overexpressing folate receptors (FR) as determined by 1PFM and 2PFM imaging experiments. The target specificity of the SiNPs was further demonstrated by three independent control experiments 1) using a FR-negative cell line, 2) testing a non-FA labeled SiNP probe on HeLa cells, and 3) blocking FR-mediated HeLa cell binding of the SiNPs with free FA.

These FR-targeting DFP-encapsulating SiNPs were demonstrated as efficient probes for *in vivo* fluorescence bioimaging upon intravenous administration into mice bearing HeLa tumors. The real-time fluorescence image monitoring for the biodistribution of the SiNPs indicated that the SiNPs selectively accumulated in the tumor, most likely via FA-mediated active targeting. Furthermore, the nanoprobe not only targeted the tumor but also penetrated deep into the tumor parenchyma as demonstrated by cellular-level 2PFM *ex vivo* imaging of whole-tumor mounts. Thus, 2PFM of the tumor provided near cell culture-image quality up to 350 μm deep in the tumor tissue. This comprehensive study of an aggregate-enhanced emission probe sets the stage for its future use in *in vivo* biomarker 2PFM imaging, in which the probe can be deliberately modified with vectors to target various tumor biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Molecular structure of DFP (left), the fluorescence emission of DFP (5×10^{-6} mol·L⁻¹) in THF and THF/water mixture (90% volume fraction of water, ca. 20 nm nanoparticle suspension) under 500 nm laser excitation (middle), and the relative fluorescence quantum yields (Φ_f) of DFP (5×10^{-6} mol·L⁻¹) vs. water fraction in THF (right).

Figure 2. (a) Zeta potential, (b) number-averaged particle size distribution by light scattering, and (c) representative TEM image (20 wt% SiNPs) of SiNPs encapsulating different amounts of DFP; the scale bar is 100 nm.

Figure 3.

(a) Fluorescence quantum yield (Φ_f) and (b) fluorescence lifetime decay of SiNPs encapsulating differing quantities of DFP.

Figure 4.

a) Fluorescence emission spectra of DFP with different concentration in PMMA and 20 wt% SiNPs dispersed in PBS (1 $\times$). b) Absorption, emission, excitation, 2PA spectra of 20 wt% SiNPs (SNP-DFP-PEGMAL) and anisotropy spectrum in PBS (1 $\times$), as well as 2PA spectra of DFP in THF.

Figure 5.

(a) Zeta potential values of surface modified DFP-doped SiNPs dispersed in water. (b) Absorption and fluorescence emission spectra of surface modified DFP-doped SiNPs dispersed in PBS. (c) ¹H NMR spectra of different surface modified DFP-doped SiNPs dispersed in D₂O, as well as SCM-PEG-MAL and Tween-80 in D₂O, FA-SH in DMSO-d₆, and DFP in CDCl₃.

Figure 6.

Images of HeLa cells incubated with SNP-DFP-PEGFA (5 μ M, 2 h) (top row), MG63 cells incubated with SNP-DFP-PEGFA (5 μ M, 2 h) (second row), HeLa cells incubated with SNP-DFP-PEGMAL (5 μ M, 2 h) (third row), HeLa cells incubated with 2 mg/mL FA 1 hour first followed by incubation with SNP-DFP-PEGFA (5 μ M, 2 h) (bottom row). Left panel: differential interference contrast (DIC), middle panel: 1PFM image, right panel: 3D reconstruction from overlaid 2PFM images (Ex: 840 nm; Power: 90 mW, ~14% on the focal plane; Em. short-pass filter 690 nm); 10 μ m grid. All images were acquired and processed with the same conditions.

Figure 7.

Representative whole-body *in vivo* fluorescence images of mice bearing HeLa tumors. The mice were intravenously administered with DFP-containing folate SiNP conjugate (left: SNP-DFP-PRGFA) or DFP-containing SiNP without folic acid derivatization (right: SNP-DFP-PEGMAL) at 3 nmol/g body weight, and the fluorescence signal was monitored at different time points post probe administration.

Figure 8.

(a) Comparison of the SiNPs delivery efficiency (organ accumulation) of the FA-conjugated silica nanoprobe and non-FA containing silica nanoprobe (Student's t test, $n = 2$ mice per group, error bars denote mean $\pm$ SEM, $*P < 0.05$) and (b) *ex vivo* image of HeLa tumor of the mice injected with SNP-DFP-PEGFA (left) and SNP-DFP-PEGMAL (right).

Figure 9.

One-photon confocal fluorescence images of HeLa tumor (~ 20 μm sections) from mice tail vein injected with SNP-DFP-PEGFA (3 nmol/g body weight, 6 h p.i.); a) bright field, b) image of the nuclei of the tumor stained with Hoechst 33285 (0.2 $\mu\text{g/mL}$), Ex: 405 nm, Em: 520 – 560 nm, DM: RSP500 nm; c) image of tumor with the channel for SNP-DFP-PEGFA, Ex: 476 nm, Em: 620 – 680 nm, DM: RSP 500 nm; d) merged image of Hoechst 33285 and SNP-DFP-PEGFA; e) localized tissue autofluorescence emission spectrum and localized fluorescence emission spectrum of the folate nanoprobe, Ex: 476 nm, Em: 500 – 760 nm, DM: RSP 500 nm; f) Higher magnification of (d) where the arrowheads indicate clusters of

the nanoprobe taken up by the tumor cells; objective 63 $\times$, NA = 1.4; representative fields from multiple sections of two tumors are shown.

Figure 10.

a) Representative 3D two-photon fluorescence images of the HeLa tumor from a mouse that was injected with SNP-DFP-PEGFA (3 nmol/g) into the tail vein; b) 50 μm deep image, c) 150 μm deep image, d) 250 μm deep image, e) 350 μm deep image, Ex: 980 nm; Power: 300 mW, $\sim 20\%$ on the focal plane; short-pass filter 840 nm, 20 $\times$ (NA = 1.0, Leica).

Scheme 1.
Synthesis and surface modification of DFP-doped SiNPs with FA surface modification.