Supplementary material (ESI) for Journal of Materials Chemistry

Supporting information

Near Infra Red Absorbing and Luminescent Gold Speckled Silica Nanoparticles for Photothermal Therapy

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Contents:

(a) Materials and Methods

Tetraethylorthosilicate (TEOS), 3-(aminopropyl)triethoxysilane (APTS), Triton X-100 (TX-100), n-hexanol, O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol, Fluorescein isothiocyanate (FITC), Isomer I, O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol [SH-Peg (5000)], heptane and cyclohexane were purchased from

Sigma Aldrich Chemical Co (St. Louis, MO). 5(6)-carboxy-Rhodamine N-succinimidyl ester (rhodamine-NHS) was obtained from Thermo Scientific (Rockford, IL). Gold chloride, and hydrazine hydrate was obtained from Acros Organics (Hampton, NH), and ammonium hydroxide (NH_4OH, 28-30 wt %) was obtained from the Fisher Scientific Co (Hampton, NH). Deionized (DI) water (NANOpure, Barnstead) was used for the preparation of all solutions: All reagents employed for the synthesis of multimodal nanoparticles were reagent grade and used without further purification.

Experimental

Preparation of GSS Nanoparticles (with and without fluorescecne): Synthesis of APTS-dye conjugate: Fluorescent core GSS were prepared by doping the silica nanoparticles with fluorescent dyes –FITC or rhodamine. Briefly, 5mg of NHS Rhodamine ester (0.008 mM) was reacted with ~10 mg of APTS in Na_2CO_3-$NaHCO_3$ buffer for up to 3h in dark conditions. For the synthesis of the FITC doped GSS nanoparticles, FITC -APTS conjugate was prepared according to reported procedure. [1]

The synthesis of the fluorescent GSS nanoparticles was done by modification of Tx-100 cyclohexane, n-hexanol (1:4.2:1 V/V) and water microemulsion[2].Briefly, 0.040 mL of TEOS, 0.030 mL of Rhodamine- APTS conjugate, 0.010 ml of APTS (for undoped silica 0.050ml TEOS) were added to the Tx-100 microemulsion, allowed to equilibrate for 30 minutes and followed by the addition of 0.100 mL NH_4OH. W_0 of the initial reaction was maintained at 10. After 24 h of stirring, $HAuCl_4$ (0.125 mL of 0.25 M in degassed water) was added in small aliquots and the microemulsion stirred for 5h. Next hydrazine hydrate (0.150 mL of 1.1M) was added in aliquots of 0.025mL at intervals of 1h and the solution stirred for 24 h. Finally the

microemulsion phase was destabilized by adding 5 ml of 200 proof ethanol and washed thoroughly with ethanol and water [2] and re-dispersed in nanopure water to obtain a concentration of ca. 1 mg/ml for further characterization. Luminescent GSS nanoparticles were protected from light during the synthesis and washing steps.

HRTEM and EDS spectra of the particles were done using JEOL 2010F Transmission Electron Microscope. The XPS of the GSS nanoparticles was done using XPS/ESCA Perkin-Elmer PHI 5100 ESCA System. UV-vis was performed using Perkin-Elmer Lambda 800 UV-Vis spectrometer.

Renishaw InVia (Hofman Estates, IL) Confocal micro-Raman Instrument was used for in vitro ablattion of cancer cells.

The XPS of the GSS nanoparticles (XPS/ESCA Perkin-Elmer PHI 5100 ESCA) on copper coated silicon wafer.

All animal experiments were approved by the University of Florida Institutional Animal Care and Use Committee.

(b) Particle size distribution.

Figure S1 shows the mean number and volume weighted particle size distribution of the GSS nanoparticles as measured by dynamic light scattering (Microtrac Nanotrac).

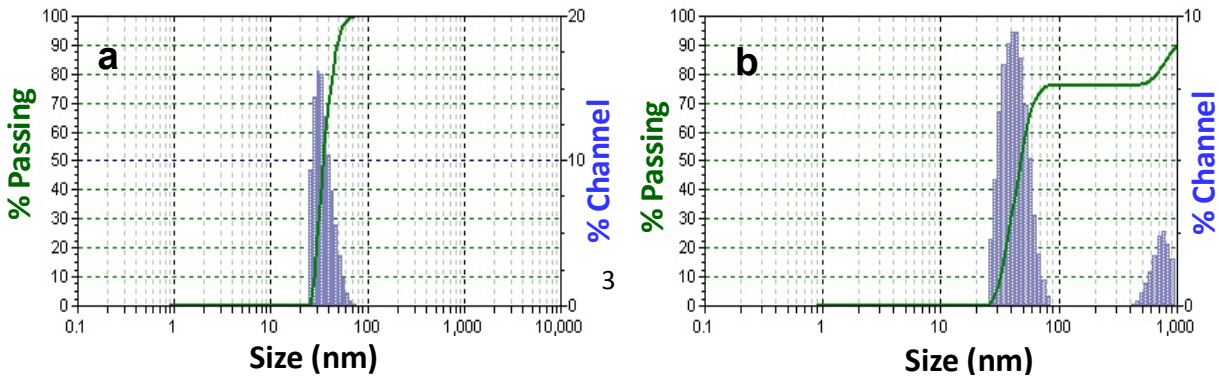

Figure S1: Shows the mean (a) number weighted and (b) volume weighted particle size distribution of the GSS nanoparticles to be ~40-50 nm. The volume distribution shows a bimodal distribution indicating the aggregation of (un-pegylated) GSS nanoparticles.

Detailed procedure for:

(i) GSS pegylation: GSS nanoparticles were conjugated with SH-PEG(5000) acid following the procedure of Bergen et al [3] for pegylation of gold nanoparticles. Briefly, ca.15 mg of the GSS nanoparticles were dispersed in degassed nanopure water with sonication. The particles were then reacted with an excess of SH-PEG(5000) at 25^{o}C for approximately 12h to conjugate to irregularly speckled gold nanodeopsits on GSS nanoparticles. The amount of PEG employed was in excess of that required to obtain ~4–5 PEG molecules nm^{-2} on gold nanoparticles[3]. The particles were separated by centrifugation and washed thrice with PBS buffer, maintaining sterile and dark conditions.

(ii) Temperature increase in solution containing GSS on NIR laser illumination

A fiber-coupled continuous wave laser source (B&W TEK Inc, BWF2-785-5-400-0.22-SMA) with a center wavelength of 785nm was used. The output power of laser was kept constant at 500 mW (350mA laser diode current) in all the experiments. An optical fiber was used to transfer the NIR laser radiation from laser source to the sample cell (FisherBrand, 1.5 ml

methacrylate cuvette, cat no. 14-955-128). The end of the optical fiber was positioned 2cm above the liquid level in the sample cell. The sample cell was filled with 1 ml of GSS nanoparticles suspension (concentration 10mg/ml) in nanopure water. A thermocouple was fixed away from the laser path in the sample cell for the temperature measurement.

(iii) Cell Culture / Plating: For this set of experiments the A549 lung cancer cell line was used (from ATCC; cell line number CCL-185) (http://www.atcc.org/) and is extensively used in lung cancer research. The growth media was prepared according the ATCC specifications (89% RPMI-1640 with L-glutamine (from Cellgro; Cat #: 25-053-CI), 10% Fetal Bovine Serum (four times filtered through 0.1 μm filter, from Hyclone; Cat. #: SH30070.03) and 1% antibiotic-antimycotic solution (from Cellgro; Cat. #: 30-004-CL)) The cells were cultured and harvested according to ATCC and the fonal cell suspension was prepared at ~3×10^6 cells/ml and viability ratio above 95%. The cell viability and concentration determination was done using a ViCell Cell counter from Beckmann-Coulter (Fullerton, CA). The cell suspention was used to plate in various culture vessels in concentration that reached 80% confluence after two days in complete media.

Nanoparticle Uptake / Imaging. A549 cells were plated in confocal dishes (from Matek Corporation; Cat. #: P35GC-1.5-14-C) following the culture guidelines established above. Cells at 80% confluency (after 24hrs of incubation) were dosed with GSS nanoparticles (20 μg/ml) and incubated for another 12 h. Four hours prior to dosing, the cells were aspirated and washed with Mg and Ca free HBSS, then incubated with 1mL of RPMI 1640 media with 1% FBS. The nanoparticles suspended in RPMI with 1% FBS were carefully administered to the A549 cells grown in the confocal dish to provide a final dose of GSS nanoparticles of 20 μg/ml

(6.23 mg/cm^2). After dosing the cells were returned to the incubator for 12hrs. Internalization of the nanoparticles were determined by confocal microscopy. Prior to the confocal microscopy experiment, the cells were rinsed with Ca and Mg free HBSS twice, to help remove free and cell surface bound nanoparticles and finally 2 ml of growth media was added to the cells. The nucleus was stained with the HOECHST 33342 agent (1 μM) approximately 1 h prior the measurement. The dye is excited by ultraviolet light at around 350 nm, and emits blue/cyan fluorescence light around an emission maximum at 461 nm when bound to DNA. For the particle tracking in the cells, the FITC enriched the particle silica core, was used. FITC has absorption maximum at wavelength 480 nm and fluorescence emission at 520 nm at pH range 5-9. An Olympus FluoView 1000 Laser Scanning Confocal Microscope System was used for all confocal imaging. Control cell cultures (without GSS nanoparticles) were used to appropriately set the confocal photomultiplier tube gains to ensure that background autofluorescence would not be registered in the images.

(iv) In vitro photothermal ablation of lung cancer cells: A549 cells were grown on MgF_2 plate (5 mm x 5mm) in a 6 well plate and dosed with GSS nanoparticles. The MgF_2 chips were then moved on a Delta T Culture Dish (from Biotechs; Cat #: 04200415C), and 2 mL of RPMI 1640 were added. The dish was placed onto a heating stage (Delta T4 Culture Dish Controller, Biotechs, Butler, PA) and temperature was maintained at 37^{0}C. The NIR laser (diode laser 785 nm, 58 mW through the 63x lens) present on the Renishaw InVia (Hofman Estates, IL) Confocal micro-Raman Instrument was scanned over the labeled cells in a nearly straight path. Next, Trypan Blue solution in phosphate buffer was added to the cells, incubated for 15

minutes and imaged using Olympus BX60 with SPOT Insight Digital Camera (Central Valley, PA, USA).

(v) In vivo photothermal ablation experiments: All animal experiments were approved by the University of Florida Institutional Animal Care and Use Committee. Six to eight week old BALB/c nude mice (CAnN.Cg-Foxn1nu/Crl) were purchased from Charles River and acclimated for 7 to 10 days. Supplemental estrogen (2μg of 17β-estradiol valerate/week) was placed subcutaneously at least 1 week prior to tumor implantation. Mice were subcutaneously inoculated with 2 x 10^7 BT474 cells on the abdominal wall. Experiments were conducted 7–10 days following implantation, after the tumors had reached approximately 10 mm in size. A volume of 300μg GSS nanoparticle suspension or control saline solution was injected into an anesthetized mouse tumor directly. The anesthetized mouse was placed on an inclined plane with the tumor exposed. Photothermal ablation experiments were carried out with 785 nm (B&W TEK Inc, BWF2-785-5-400-0.22-SMA), continuous wave laser at 500 mW output power (power density 325mW/cm^2) for 10 minutes. The distance between the optical fiber and the tumor was maintained at 15 mm for all *in vivo* experiments. The mice were recovered for 20 hours and then sacrificed using approved methods. Tumors were harvested and fixed in formaldehyde solution. Tissue sections were stained with Hematoxylin and Eosin and subsequently analyzed. Percentage of necrosis was analyzed in tumor specimens in treated and control tumors by pathologists blinded to the treatment

Tissue handling and preparation for fluorescence microscopy: Tumor tissue fixed in 4% paraformaldehyde (PFA) by reperfusion fixation at 4°C, and subsequent immersion for 6 hours at room temperature, then washed in phosphate-buffer saline (PBS) for 48 hours at 4°C. They

were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and stored at -80°C for frozen tissue specimens until further analysis. Sections were rehydrated in PBS and stained by 10μM HOECHST 33342 (AnaSpec, CA). Then, the tissue observed under fluorescence microscope (Olympus IX71).

(vi) Au estimation by Inductively Coupled Plasma (ICP) elemental analysis. ICP measurements were performed using Perkin-Elmer Plasma 3200 system equipped with two monochromators covering the spectral range of 165-785 nm with a grated ruling of 3600 lines/mm. The ICP estimation of gold was done by digestion of GSS nanopartilces with aqua regia. Briefly, 0.010g of the dry GSS nanoparticles were digested using aqua regia solution [Aqua regia digestion should be performed with care in a hood.]. Au was completely solubilized in the oxidizing acidic media while the silica matrix separated out as white powder. The solution was filtered to separate the silica particulate residue. The residue was washed with aqua regia solution and finally twice with nanopure water. The diluted filtrate was concentrated, by boiling and the final volume was reduced to 20.0 mL. The instrument was calibrated with gold standard solutions followed by estimation of Au (wavelengths -242.795 nm and 267.595 nm).

(d) Quantitative uptake of GSS nanoparticles by cancer cells using Flow cytometry

The flow cytometry experiments were done following the same nanoparticle dosing protocols as above. Briefly, cells (3.0×10^6/well) were seeded into 6 well tissue culture plates and allowed to adhere for 24 hours. Cells were exposed to FITC doped PEGylated GSS nanoparticles (20μl or 40μl) for 24 h. Cells were harvested by 0.25% trypsin with EDTA, washed with PBS, centrifuged (at 500 g for 5 min) and resuspended in PBS. Uptake of FITC doped PEGylated GSS

was analyzed using a BD LSR flow cytometer (Becton Dickinson Biosciences, CA) with computer-assisted data analysis (BD FACSDiva). At least 10,000 cells were analyzed in each sample and all experiments were performed at least in duplicate (Fig**. S2**). The uptake of the pegylated GSS nanoparticles by the BT474 cell line can be explained by the heterogeneous surface of the nanoparticles. The chemical route used in this study selectively attaches peg to the randomly and irregularly present gold nanodomains, leaving the silica surface largely uncoated. Thus forming, a non uniform pegylated surface that likely does not completely inhibit protein adsorption nor particle endocytosis during the 24h incubation time.

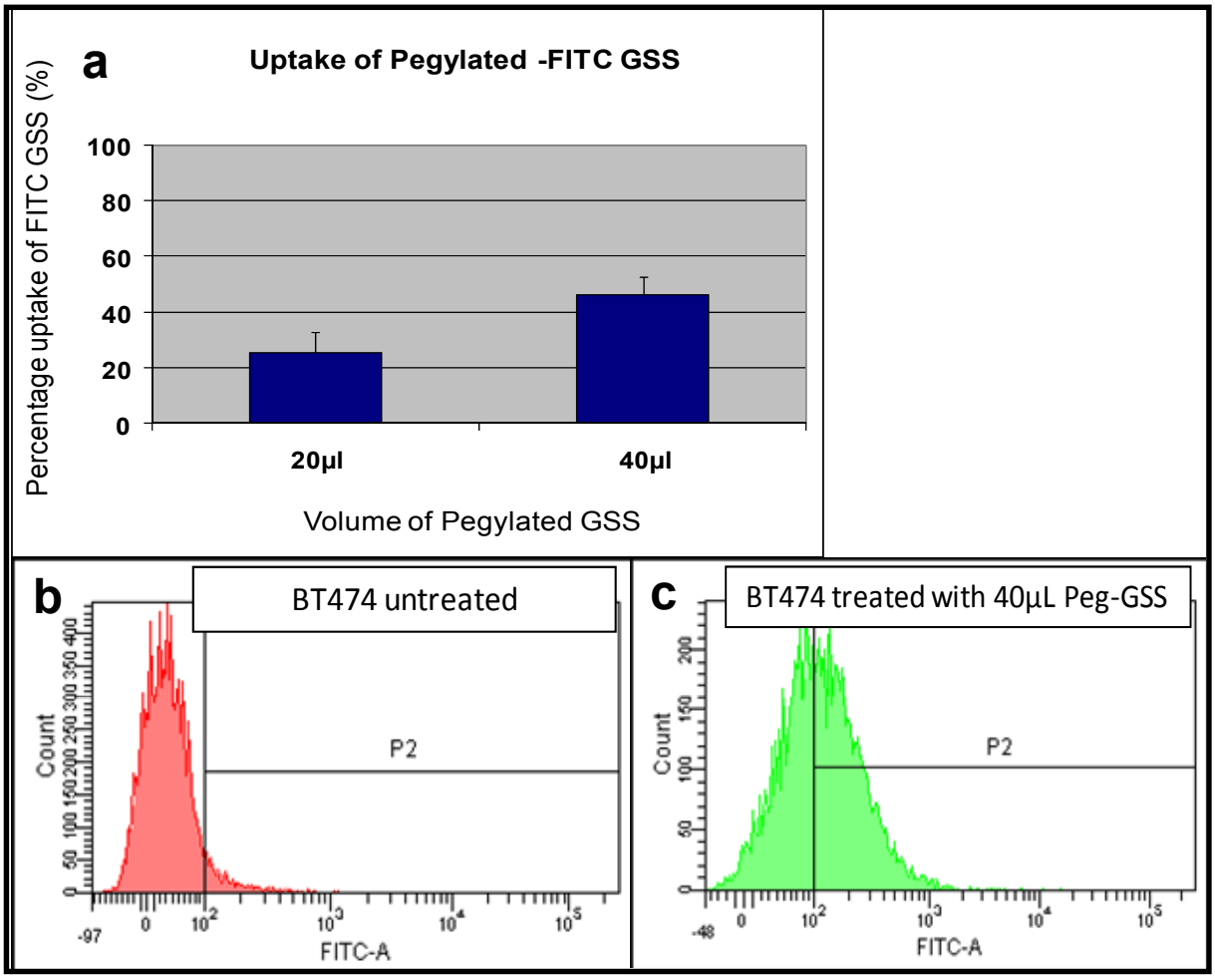

Figure S2. Shows the increase in uptake of Pegylated GSS nanoparticles by breast cancer BT474 cells at two different doses (20µL and 40µL of 1mg/mL); (b) and (c) show the representative FACS spectra of untreated and labeled cells respectively.

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