Dual Imaging Probes for Magnetic Resonance Imaging and Fluorescence Microscopy Based on Perovskite Manganite Nanoparticles

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Supplementary Information

SI-1. Contents

SI-2. Detailed experimental section

SI-2.1. Materials and methods

Fluoresceinisothiocyanate (FITC) was prepared according to the published procedure[S1] by reaction of fluoresceinamine and thiophosgene. Tetraethoxysilane (TEOS), 3-aminopropyltriethoxysilane (APS), fluoresceinamine (isomer I, ≥95 %), thiophosgene, polyvinylpyrrolidone (PVP) K15 (M_r = 10 000), K25 (M_r = 24 000) and K90 (M_r = 360 000) were all purchased from Aldrich (Milwaukee, USA). $SrCO_3$, $MnCO_3$, La_2O_3, citric acid monohydrate and conc. H_2O_2 were purchased from Fluka (Buchs, Switzerland). HNO_3, conc. ammonia and ethyleneglycol were purchased from Penta (Chrudim, Czech Republic). Metal contents in the starting materials were determined by means of chelatometric titration.

Phase composition and the mean size of crystallites d_{XRD} were analyzed by XRD (Bruker D8 Advance, CuKα, Sol-X energy dispersive detector). The content of Mn in the samples was determined spectrophotometrically after its oxidation to MnO_4^-. The details for these analyses are described in the previous paper.[23] Magnetic properties of dried samples (washed with ethanol and dried in vacuum drier at 40 °C) were studied by means of a SQUID magnetometer (Quantum Design MPMS-5S) in fields up to 1200 kA·m^{-1}. The Curie temperature T_C of the samples was evaluated using Arrott plot. Morphology and size of the particles was investigated by means of TEM (Philips EM 201; 80 kV, W filament) and HRTEM (Jeol JEM 3010; 300 kV, LaB_6 tip), for which a drop of diluted sample was placed on a carbon coated copper grid (SPI 3630C–MB) and was left to dry freely. In order to determine the thickness of silica layers, the image analysis of TEM micrographs was carried out in ImageJ 1.37a.[S2]. The spherical approximation was adopted requiring that the cross-section of the model is equal to the average area found by image analysis. Photon Correlation Spectroscopy (PCS) was employed for hydrodynamic size determination using the measuring system composed of 633 nm He–Ne laser system, goniometer ALV CGS/8F (measurement at the angle of 90°), detector ALV High QE APD and multibit multitau autocorrelator ALV 5000/EPP. The highly diluted sample in glass cuvette was shortly sonicated in ultrasound bath prior to the measurement. Surface ζ-potential was studied by means of Malvern Autosizer Lo-C instrument. The measurement was performed at 25 °C on a set of diluted samples with pH = 1–12 that was adjusted by addition of NaOH or HCl solutions and measured by pH-meter Radiometer pIONeer (glass electrode Mettler Toledo).

IR spectra were measured on the FTIR spectrometer Nicolet Magna 760 (4 cm^{-1} resolution, Happ-Genzel appodization) employing DRIFTS method. The samples were dried in the vacuum drier at 40 °C, stored in a desiccator over KOH and before measurement gently homogenized with optically pure KBr. Fluorescence spectra were recorded using the AMINCO Bowman Series 2 FA–357 luminescence spectrometer on diluted samples in quartz fluorescence cuvettes Hellma 111–QS at 25 °C. MS spectra of low molecular intermediates were acquired on Bruker spectrometer ESQUIRE 3000 equipped with an electrospray ion source and ion trap in positive/negative mode. ^{1}H NMR spectra of intermediate ethanol solution of fluorescent alkoxysilane were measured in DMSO-d^6 (Aldrich, Milwaukee, USA) on spectrometer Varian ^{UNITY}*INOVA* 400 at 25 °C.

Measurements of temperature dependence of the spin-spin relaxation time T_2 at a magnetic field $B_0 = 0.5$ T (20 MHz) was carried out on a relaxometer Bruker MiniSpec using a standard CPMG sequence[S3] with following parameters: echo spacing T_E' = 2 ms, repetition time $T_R = 5$ s, number of acquisitions $AC = 8$. The number of echoes varied according to actual T_2 to cover at least a time interval of $10T_2$. The T_2 values were obtained by fitting the signal I with formula $I = a \cdot \exp(-T_2/T_E) + b$. The temperature of the probe was stabilized by an external thermostat Hakke C10 and the temperature was measured directly in the sample. MR spin-spin relaxometry at higher magnetic fields was performed at 20 °C using the 1.5 T Siemens MAGNETOM Avanto and the 3 T Siemens MAGNETOM TrioTrimm spectrometers. A standard CPMG (repetition time $T_R = 5$ s, $T_{Effective} = 6.9$ ms, number of echos = 32, slice thickness = 20 mm, number of acquisitions $AC = 64$, $FOV = 180 \times 90$ mm^2, matrix = 128×128) was used. The values of spin-spin relaxivity r_2 in all the measurements were determined according to the formula: $r_2 = [1/T_2 - 1/T_{20}]/c_{Mn}$, where T_2 is the relaxation time of the sample, T_{20} is the relaxation time of pure water and c_{Mn} is the molar concentration of manganese being 55 μmol·L^{-1} in all the experiments.

SI-2.2. Synthesis of fluorescent alkoxysilane (FITC-APS)

The synthesis was carried out under the argon atmosphere in the dark. The anhydrous ethanol (10 mL), freshly dried by means of magnesium ethanolate,[S4] was distilled to the argon-rinsed flask containing FITC (110 mg, 0.282 mmol). After the dissolution of FITC, APS (50 μL, 0.238 mmol) was added. The mixture was magnetically stirred for 24 h and then, a part of the reaction mixture was immediately used for preparation of LSMO@siF without any separation of FITC-APS. The conversion was approximately 50 % (according to integration of ^{1}H NMR spectra). Positive and negative MS spectra confirmed the presence of the desired product (molecular peak of FITC-APS) while TLC exhibited the presence of the product and both the reactants.

MS (ES, positive): *m/z* (%): 633.2 (100) [(M+Na)$^+$], 222 (58) [APS$^+$], 390 (28) [FITC$^+$], 611 (25) [(M+H)$^+$)]; MS (ES, negative): *m/z* (%): 609(100) [(M–H)$^-$)], 610(37) [M$^-$]; 611(16) [(M+H)$^-$]

TLC (Merck Silica Gel 60 F_{254}; THF: toluene 1:1): $R_f = 0.6$, visual detection (FITC-APS); $R_f = 0.0$, ninhydrin detection (APS); $R_f = 0.7$, visual detection (FITC).

SI-2.3. Preparation of $La_{0.75}Sr_{0.25}MnO_3$ nanoparticles (LSMO)

The nanoparticles were synthesized similarly to the previously described citrate procedure from commercially available materials taking into account the chemically determined content of the metals.[9,22,23] The starting solution was prepared from La_2O_3 (4.887 g, 7.5 mmol) and $SrCO_3$ (0.738 g, 5.0 mmol) using HNO_3 (10 mL, 1:1), while $MnCO_3$ (2.229 g, 20 mmol) was dissolved separately in HNO_3 (10 mL, 1:1), providing small amount of MnO_2 in form of a brown precipitate. The precipitate was dissolved by addition of small amount of concentrated H_2O_2 and the solution was heated up to the

boil for several minutes on a hot-plate. After mixing of both the solutions, citric acid monohydrate (12.61 g, 60 mmol) was added and the mixture was stirred for 1 h. The ammonia was used to adjust pH to 7.6 and then, ethyleneglycol (5.59 g, 90 mmol) was added. Further stirring and slow heating enabled gradual evaporation up to complete drying of the organic precursor that was burned at approximately 200 °C. The following thermal treatment involved calcination at 400 °C for 4 h and annealing at 750 °C for 3 h, both in the air atmosphere. The raw as-grown product was subjected to three cycles of the rolling (simple rolling machine with the gap of $\approx 10^{-2}$ mm) followed by the ball milling (Retsch MM 301, 25 cm^3 WC milling vessel, WC ball with diameter of 1.5 cm) in ethanol at frequency of 21 Hz for 1 h. The XRD study confirmed single phase composition and provided the mean size of crystallites d_{XRD} = 20 nm (see Fig. S2). IR spectra of product corresponded to previously reported data.[9,22]

SI-2.4. Biological studies

SI-2.4.1. Labeling of HeLa cells and fibroblasts

HeLa cells and human normal skin fibroblasts were incubated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Scotland) supplemented with 10 % fetal bovine serum (FBS, Gibco BRL, Scotland), 40 $\mu g \cdot L^{-1}$ gentamycin (Lek, Slovenia), 0.25 $mg \cdot mL^{-1}$ glutamine (Sevapharma, Czech Republic) and with different concentrations of the nanoparticles (37 °C, 5 % CO_2, 48 h). Both adherent cells types were harvested using 0.5 mL of trypsine/EDTA solution in PBS. Cells released into suspension were mixed with the nonadherent ones floating in the incubation medium collected previously. The mixture was stained with propidium iodide for distinguishing the living and the dead cells and analyzed by the flow cytometry (LSR II, Becton Dickinson) measuring the mean fluorescence intensity of the cells in FITC channel as well. The numbers of events for the unambiguously living N_L and the unambiguously dead cells N_D were recorded, whereas the viability was determined as the $(N_L/(N_L+N_D))\cdot 100$ % ratio. For the fluorescence microscopy cells were incubated under the same cultivation conditions on cover slips. For the experiment cells on coverslips were rinsed with PBS twice and fixed with 3.8 % formaldehyde for 20 min directly in the multiwell plate. Fixation was followed by washing with PBS, neutralization with 0.015 M NH_4Cl and finally washing twice with PBS. The cover slips were mounted in mowiol containing 4',6-diamidino-2-phenylindole (DAPI) (1 μg/50 mL) and the specimens were imaged using the Olympus IX-81 CellR microscope equipped with Hamamatsu C4742-80-12AG digital camera under oil-immersion lens 63× and Olympus U-M3DAFITR emission and excitation filters for DAPI and FITC.

SI-2.4.2. Labeling of rMSCs

Rat bone marrow mesenchymal stem cells (rMSCs) were obtained from the tibia and femur of 4-week-old Wistar rats. The ends of the bones were cut and the marrow was extruded with DMEM (PAA Laboratories, Linz, Austria).[S5] Marrow cells were plated in a 75-cm^2 tissue culture flask (TPP,

Trasadingen, Switzerland) in DMEM medium containing 10 % FBS (PAA Laboratories, Linz, Austria), 100 units·mL^{-1} penicillin (Gibco BRL, Paisley, Scotland), and 0.1 mg·mL^{-1} streptomycin (Gibco BRL, Paisley, Scotland). After 24 h, the nonadherent cells were removed by replacing the medium. Cells from passage 2–5 were used for following experiments. Cells were inoculated into 12 well plates, 100 000 cells per well and incubated with different concentration of nanoparticles in culture media for 48 h. At the end of the labeling experiment, all cells were harvested with trypsin/EDTA and counted in a Bürker chamber. Live and dead cells were distinguished using the trypan blue exclusion test.[S6] Viability was calculated as a ratio $(1–N_D/N)\cdot 100$ %, where N_D is number of blue stained dead cells whereas N is number of all cells in Bürker chamber. Nanoparticles were visualized in the cells using inverted fluorescent microscope Zeiss Axiovert 200 equipped with digital camera Axiocam MRc5.

SI-2.4.3. Labeling of pancreatic islets

Pancreatic islets (PI) were isolated from adult male Lewis rats. The islets were incubated in CMRL-1066 medium (PANBiotech, Aidenbach, Germany) with 10 % fetal serum, 1 % penicilin/streptomycin/glutamin and 1 % HEPES (all Sigma). And then they were incubated at concentration of LSMO@siF@si-w corresponding to manganese content of 0.11 mmol(Mn)·L^{-1} for 24 h.

The vitality of PIs was tested using the fluorescent markers propidium iodide and acridine orange. Propidium iodide enters dead or dying cells and stains their nuclei red; acridine orange enters in low concentrations the living cells and colours their cytoplasm green. Stained PIs were grouped under fluorescence microscope (Olympus BX41) according to the ratio of dead cells into groups with 0−25, 25−50, 50−75 and 75−100 % and thereout the overall vitality was calculated.

The ability to produce insulin was tested using a static incubation technique. The base solution was prepared by mixing equal amounts of Krebs I (NaCl 26.892 g·L^{-1}), Krebs II (KCl 1.490 g·L^{-1} + $NaHCO_3$ 8.064 g·L^{-1} + $MgCl_2$ 0.814 g·L^{-1}) and Krebs III ($CaCl_2$ 1.11 g·L^{-1}) solutions. Into the base solution, glucose was added to reach the concentration of 3.3 mM, while the stimulation solution was prepared by dissolving glucose to the concentration of 22 mM. PIs were cultivated for 60 min in the base solution, then in the stimulation solution for the next 60 min and subsequently for 60 min again in the base solution. After each incubation period, the concentration of insulin was determined using a ^{125}I RIA Kit (ICN Pharmaceuticals). The stimulation index (*SI*) was calculated as the ratio of the insulin concentration after the stimulation and the insulin concentration in the first non-stimulating media.

The uptake of the LSMO@siF@si-w nanoparticles into PIs was investigated by means of immunofluorescence detection. Labeled islets were fixed with 4 % formaldehyde and embedded in paraffine. The immunoflurescence staining was performed on 5 μm-sections. After blocking the non-specific binding sites with 5 % donkey serum the specific antibodies against c-peptide and

macrophages were applied, namely the rabbit anti-human c-peptide and mouse anti-rat monocytes/macrophages antibodies (Millipore). Anti-rabbit and anti-mouse secondary antibodies conjugated with AlexaFluor 555 (Invitrogen) were applied and the nuclei were counterstained by DAPI (Sigma). The sections were mounted in Dabco-Mowiol solution in glycerol and imaged using Olympus BX41 fluorescence microscope equipped with Olympus DP71 digital camera. The presence of the nanoparticles was detected in green channel.

Gel samples for MR imaging were prepared by placing 5–80 labeled PIs in a well (diameter 35 mm), in one layer sandwiched between two layers of gelatin, the bottom layer being of 4 % gelatin and the top layer of 3% gelatin. The MR measurements were performed using a 4.7 T Bruker Biospec spectrometer equipped with a commercially available resonator coil (Bruker) at 25 °C. A standard T_2-weighted CPMG multispin echo sequence (repetition time $T_R = 3$ s, $T_E = 36$ ms, slice thickness = 0.63 mm, turbo factor = 8, number of acquisitions = 1–64, $FOV = 4\times4$ cm^2, matrix = 256×256) and T_2^*-weighted gradient echo (repetition time $T_R = 128$ ms, $T_{Effective} = 3.7$ ms, slice thickness = 0.63 mm, number of acquisitions $AC = 128$, $FOV = 4\times4$ cm^2, matrix = 256×256) were used.

SI-3. Supplementary figures

Fig. S1 Photo of LSMO@siF@si-w water dispersion under daylight (left) and under UV irradiation (right).

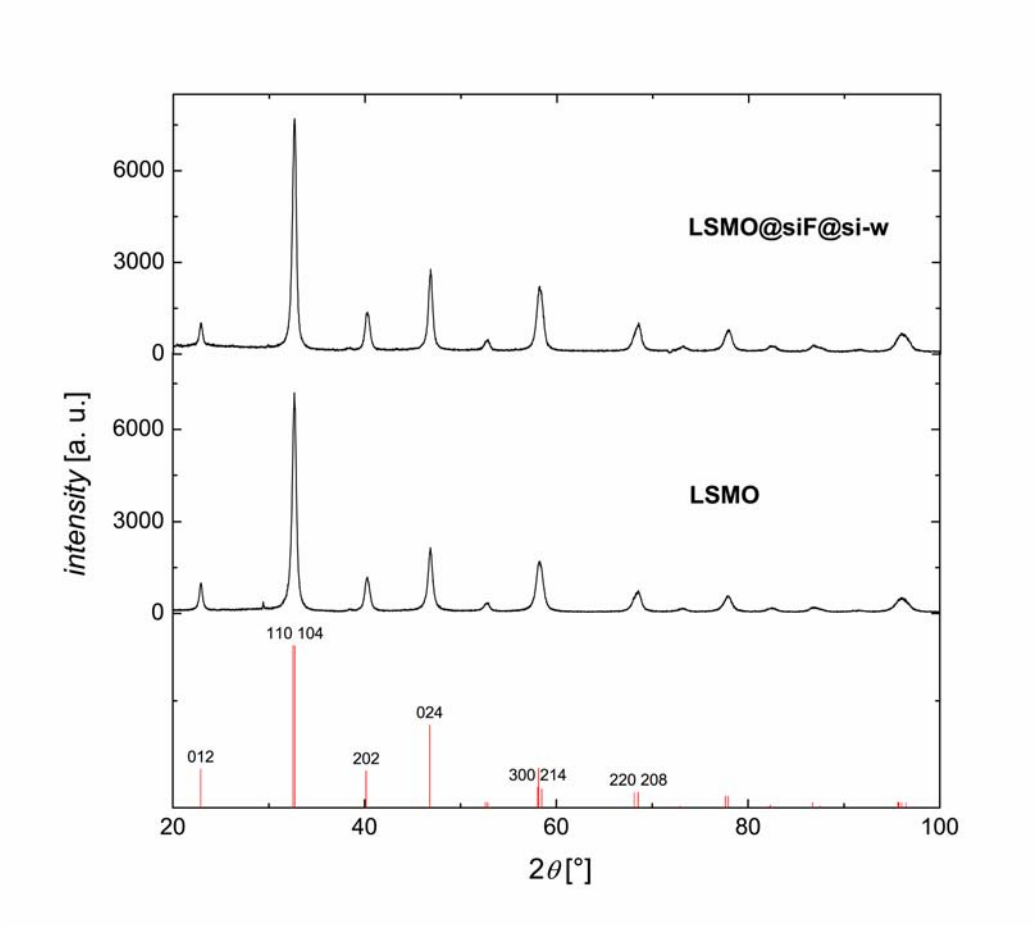

Fig. S2 XRD patterns of the LSMO nanoparticles and the resulting LSMO@siF@si-w product (prepared from the same batch of LSMO).

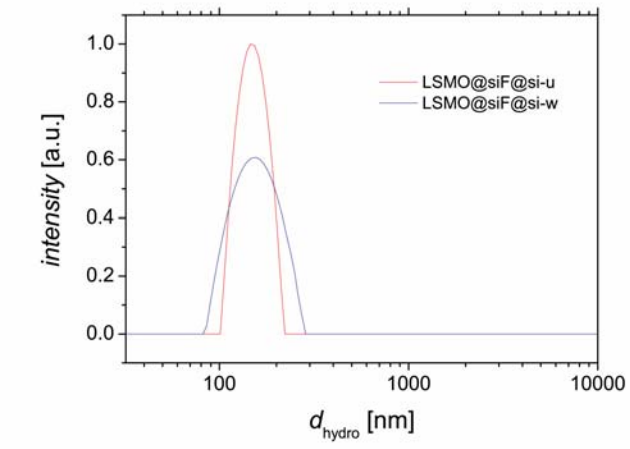

Fig. S3 The hydrodynamic diameter d_{hydro} of the final products and the distribution of the signal intensity in PCS.

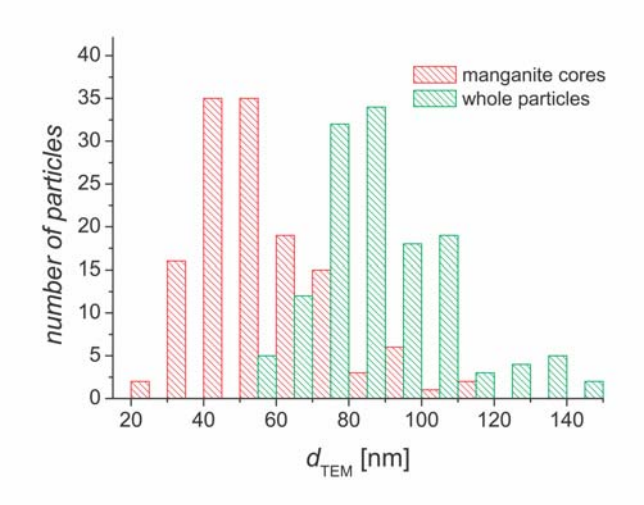

Fig. S4 Image analysis of TEM micrographs for LSMO@siF@si-u sample showing the overall particle diameter and the diameter of its magnetic core.

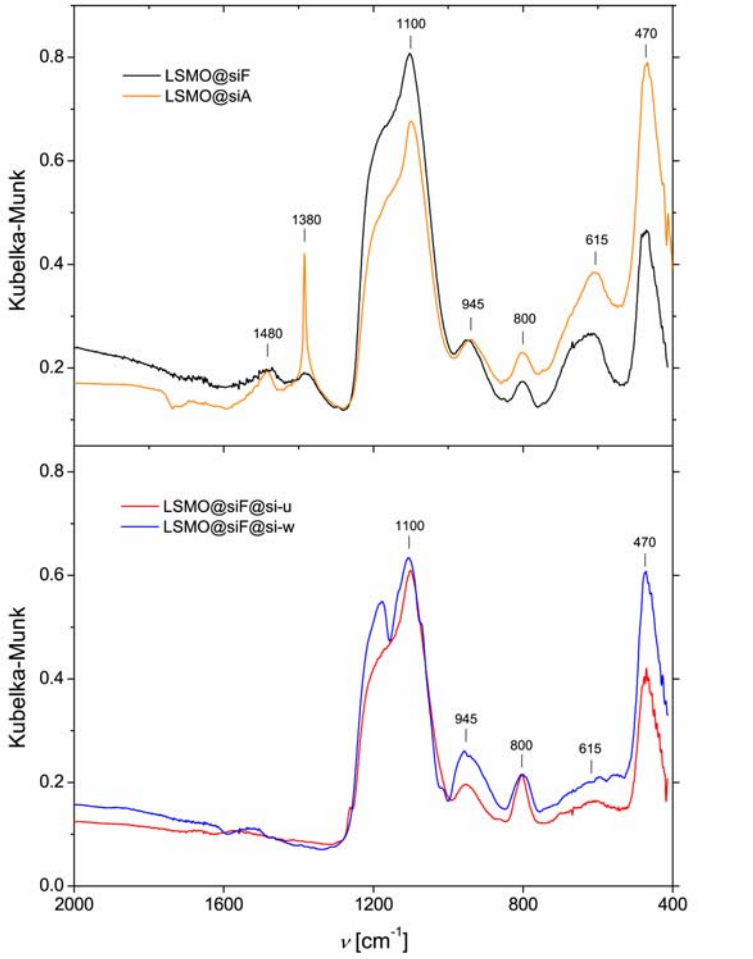

Fig. S5 IR spectra of both the products with two-ply shell (LSMO@siF@si-u and LSMO@siF@si-w), the intermediate without the outer layer (LSMO@siF) and the sample without fluorescein (LSMO@siA) measured by the DRIFTS method.

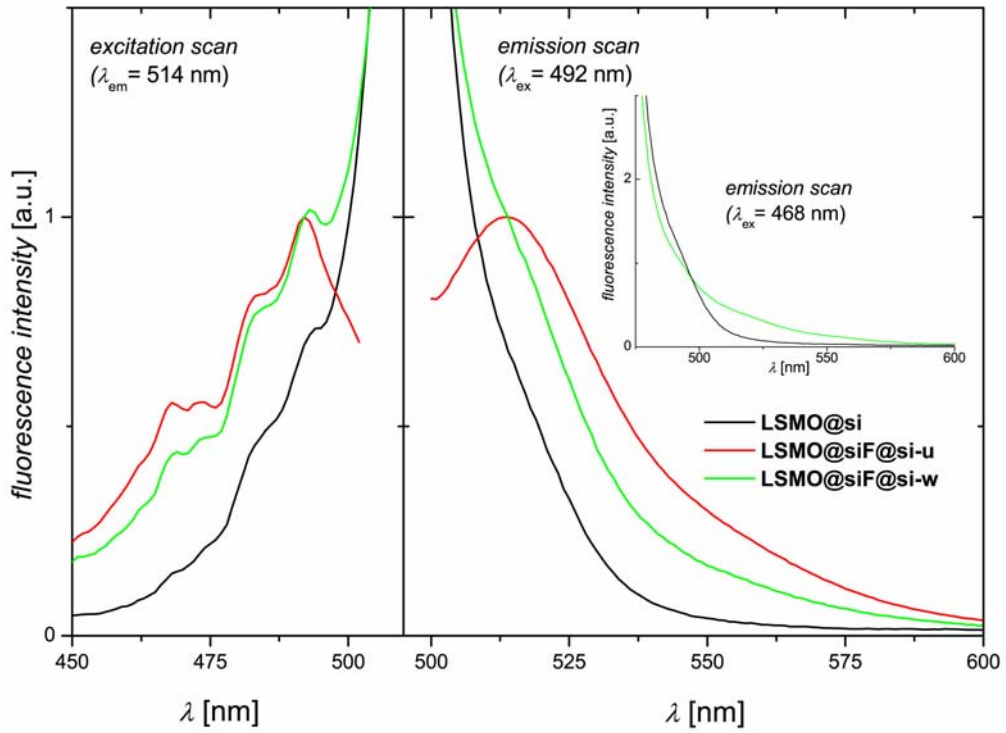

Fig. S6 Fluorescence spectra (excitation scan at $\lambda_{em} = 514$ nm and emission scan at $\lambda_{ex} = 492$ nm) of pure silica coated LSMO particles (LSMO@si) and both the LSMO@siF@si-u and LSMO@siF@si-w products with the inset showing emission scan at $\lambda_{ex} = 468$ nm.

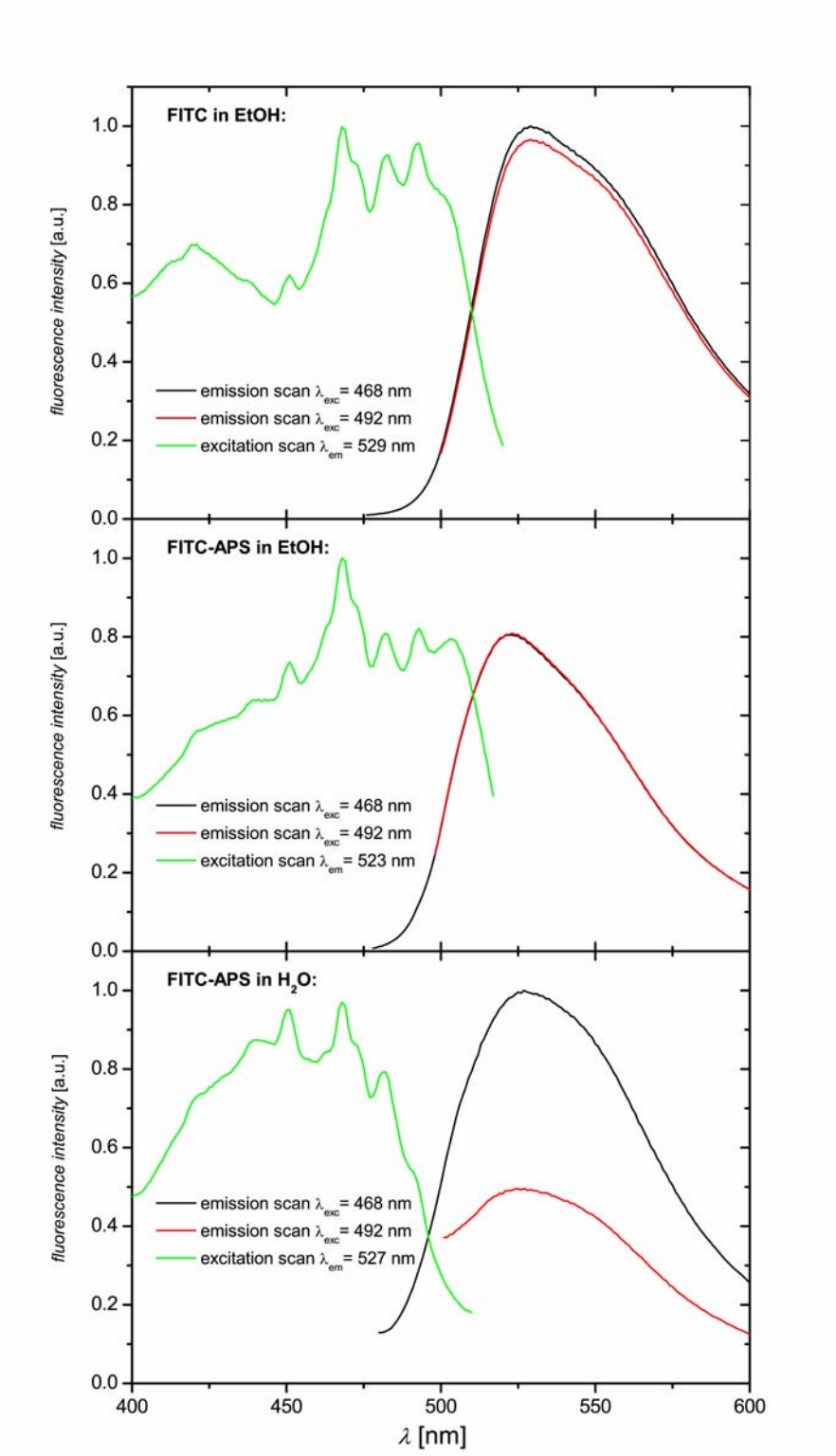

Fig. S7 Fluorescence spectra (excitation and emission scans) of pure FITC in anhydrous ethanol, the reaction mixture of FITC-APS in anhydrous ethanol and the hydrolyzed mixture APS-FITC in water.

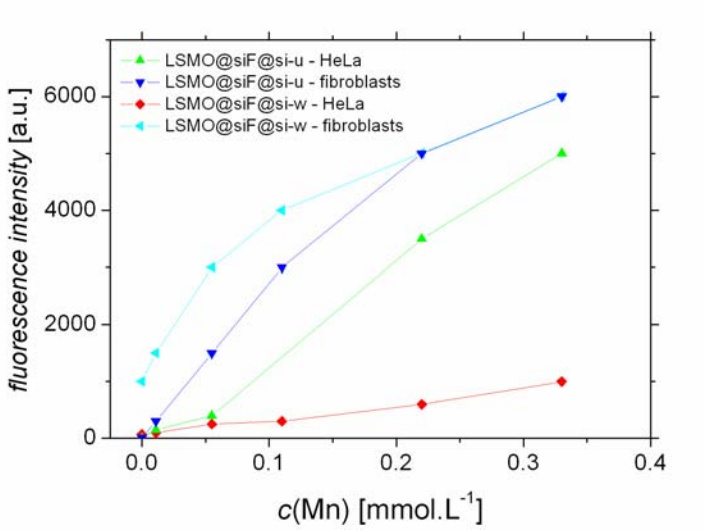

Fig. S8 Mean fluorescence intensities of the cells measured by flow cytometry in FITC channel.

SI-4. References

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