Electronic Supplementary Information (ESI)

A glucose-depleting silica nanosystem for increasing reactive oxygen species and scavenging glutathione in cancer therapy

Wee Kong Ong,[a] Deblin Jana,[a] and Yanli Zhao[a,*]

[a]Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore, 637371.
Email: zhaoyanli@ntu.edu.sg

Materials

All solvents and chemicals were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Alfa Aesar. MDA-MB-231 cells were obtained from ATCC.

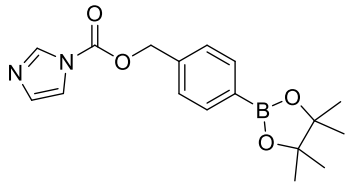
Characterizations

^{1}H NMR spectra were obtained on 500 MHz Bruker spectrometer using deuterated DMSO as the solvent. X-ray photoelectric spectroscopy (XPS) was carried out on a Phoibos 100 spectrometer. N_2 adsorption/desorption analyses were conducted using Quantachrome Instruments Autosorb-iQ (Boynton Beach, Florida USA) with extra-high pure gases. Specific surface areas were calculated from the adsorption data in low-pressure range using the Brunauer−Emmett−Teller (BET) model, and pore size was determined by following the Barret−Joyner−Halenda (BJH) method. Thermogravimetric analysis (TGA) was carried out for powder samples using a TGA Q500 recorded from 100 −800 °C in an air flow at a heating rate of 10 °C min^{-1}. Powder X-ray diffraction (XRD) patterns were collected using Shimadzu XRD-6000e quipped with Cu-Ka radiation (λ = 1.5418 Å). Fourier transform infrared (FTIR) spectra were recorded as KBr-pellet on a SHIMADZU IR Prestige-21 spectrophotometer. Transmission electronic microscope (TEM) images were collected on a JEM-1400 (JEOL) and JEOL 2010 UHR operated at 100 kV. Zeta potential and dynamic light scattering (DLS) measurements were measured using Zetasizer, Malvern Instrument Ltd. UV-vis-NIR absorption and fluorescence emission spectra were performed on a Shimadzu UV-3600 and Shimadzu RF5301PC spectrophotometer, respectively. Confocal laser scanning microscopy (CLSM) images were acquired by a Carl ZEISS LSM 800. Flow cytometry was recorded on a BD LSR Rortessa X20 (3 lasers) and BD LSR Rortessa X20 (5 lasers).

Synthesis procedures

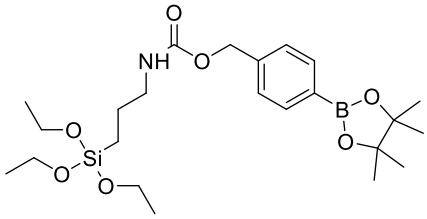
Schematic synthetic procedures of CDI-PE and bornate ester-protected quinone methide silane ligand (Si-PE) are shown in Schemes S3 and S4.

Synthesis of CDI-PE



CDI-PE was synthesized according to a reported procedure.[1] 4-(Hydroxymethyl)phenylboronic acid (1 g, 4.27 mmol) was dissolved in anhydrous dichloromethane (10 mL). Carbonyldiimidazole (CDI) was subsequently added and the mixture was stirred for 2h. The mixture was then evaporated and re-dissolved in ethyl acetate (15 mL), followed by washing with deionized water (3 × 25 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered, and concentrated to afford CDI-PE as a white solid (1.22 g, yield: 87%). ^{1}H NMR (300 MHz, δ, ppm, DMSO-d_6): 8.30 (s, 1H), 7.71 (d, 2H), 7.62 (d, 1H), 7.51 (d, 2H), 7.09 (d, 1H), 5.47 (s, 2H), 3.31 (s, 2H), 1.45 (m, 2H), 1.30 (s, 12H). ^{13}C NMR (400 MHz, δ, ppm, DMSO-d_6): 148.7, 138.4, 137.8, 135.1, 130.9, 127.9, 118.0, 84.23, 79.5, 69.4, 25.1.

Synthesis of Si-PE



CDI-PE (0.610 g, 1.86 mmol) was dissolved in anhydrous dichloromethane (10 mL). Dimethylaminopyridine (2.2 mg, 0.01 eq) and (3-aminopropyl)triethoxysilane (513 mg, 1.3 eq) were subsequently added and the mixture was stirred at room temperature for 24h. The mixture was then evaporated and purified by column chromatography (silica gel, hexane/ethyl acetate (10:1, v/v)) to afford Si-PE as a yellow oil (0.45 g, yield: 50%). ^{1}H NMR (300 MHz, δ, ppm, DMSO-d_6): 7.65 (d, 2H), 7.33 (d, 2H), 7.28 (t, 1H), 5.03 (s, 2H), 3.72 (q, 6H), 3.31 (s, 9H), 2.96 (q, 2H), 1.45 (m, 2H), 1.29 (s, 12H), 1.13 (t, 10H), 0.51 (m, 2H). ^{13}C NMR (400 MHz, δ, ppm, DMSO-d_6): 156.47, 141.26, 134.92, 127.22, 127.18, 84.12, 58.14, 56.49, 25.42, 25.13, 19.02, 18.66. HRMS (EI): m/z $[M]^+$ calcd. for $C_{23}H_{40}BNO_7Si$: 481.27, found: 482.2751.

Synthesis of monodispersed organically-modified silica nanoparticle (ORMOSIL)

The synthesis of ORMOSIL followed a modified Stöber process.[2] Typically, CTAB (0.125 g) was placed in a 250 mL round bottom flask containing ultrapure water (60 g) and a solution of 2M NaOH (0.44 mL). This solution was heated to 70 °C under vigorous stirring (750 rpm). After 1 hour of stabilization, an initial amount of TEOS (0.1 mL) was added to the mixture. After 30 min of the reaction, additional TEOS (0.5 mL) and Si-PE (130mg) were added to complete the reaction. The condensation process was conducted for 1.5 h. After the reaction, the mixture was slowly cooled down at room temperature while stirring, and then collected in

Nalgene™ tubes for centrifugation at 9000rpm for 10 min. To extract the surfactant from the nanoparticles, the solid was redispersed in a 100 mL round bottom flask containing concentrated 37% HCl (8mL) in methanol (50mL), and was refluxed at 80 °C for 24h. The nanoparticles were then collected by centrifugation and washed with water and ethanol twice respectively before vacuum dried at 50°C overnight.

Synthesis of FITC-labelled glucose oxidase (FITC-GOx)

FITC-GOx was prepared according to the reported method.[3] Briefly, FITC in DMSO (25 μL, 10 mg/mL) was added into GOx aqueous solution (1 mL, 10 mg/mL). After incubation at 4 °C for 12 h, the reaction mixture was dialyzed against deionized water overnight in the dark, followed by lyophilization. FITC numbers conjugated to GOx were determined by the extinction coefficient of 81,000 M^{-1} cm^{-1} at 495 nm (FITC). The number of conjugated FITC per GOx was estimated to be 4.2.

Synthesis of GOx-conjugated ORMOSIL (ORMOSIL@GOx)

GOx (4 mg) was first dissolved in deionized water (4 mL). An aliquot (1 mL) of this GOx stock solution was then added into an aqueous solution consisting of deionized water (2 mL), EDC (20 mg), NHS (30 mg) and APTES (23 μL), which was then stirred at room temperature for 8h. A well-sonicated suspension of ORMOSIL (10 mg) was subsequently added into the amino-functionalized GOx and stirred at room temperature for 24h to form ORMOSIL@GOx. ORMOSIL@GOx was washed thrice with deionized water to remove the unreacted GOx.

Cell culture

MDA-MB-231 cells were cultured in DMEM supplemented with 10% (v/v) FBS, *L*- glutamine (0.03%) and 1% penicillin-streptomycin under 5% CO_2 atmosphere at 37 °C. DMEM was replaced every two days.

***In vitro* cytotoxicity assay**

The anticancer efficiency of SiNP, GOx, ORMOSIL and ORMOSIL@GOx was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the metastasis MDA-MB-231 breast cancer cells. Briefly, cancer cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM (100 μL) containing 10% FBS and 1% penicillin at 37 °C in 5% CO_2 humidified atmosphere. After 24 h incubation, the medium was replaced with fresh one (85 μL) and doped with glucose (5μL, 20 mg/mL), followed by the addition of free GOx, ORMOSIL or ORMOSIL@GOx at various concentrations of GOx (1-10 mU/mL). After 24h incubation, the medium was again replaced with fresh one (90 μL), followed by the addition of MTT solution (10 μL, 5 mg/mL in PBS buffer). The plate was incubated further for 4 h, before the medium was removed and replaced with DMSO (100 μL) to dissolve the purple formazan crystals. Finally, the optical density of the sample in each well was analyzed at 490 nm by a microplate reader (infinite M200, TECAN). The cell viability (%) was calculated based on the following equation, $(A_{sample}/A_{control}) \times 100\%$, where A_{sample} and $A_{control}$ represent the absorbance of the sample and control groups, respectively.

Intracellular ROS and GSH quantification

MDA-MB-231 breast cancer cells were seeded at a density of 2×10^5 cells per well into a 8-well glass plate and incubated in DMEM high glucose medium (200μL) with 10% FBS and 1% penicillin at 37 °C with 5% CO_2 humidified atmosphere for 24h. The medium was replaced with fresh DMEM containing glucose (1 mg/mL), followed by the addition of free GOx, ORMOSIL, or ORMOSIL@GOx at GOx concentration of 7.5 mU/mL. After 24 h, ROS probe (DCFH-DA) was added at the final concentration of 10 μM and incubated for 20 min. Then, the cells were directly imaged with CLSM. DCF decomposed from DCFH-DA was excited at 488 nm, and fluorescence was detected from 500 to 550 nm.

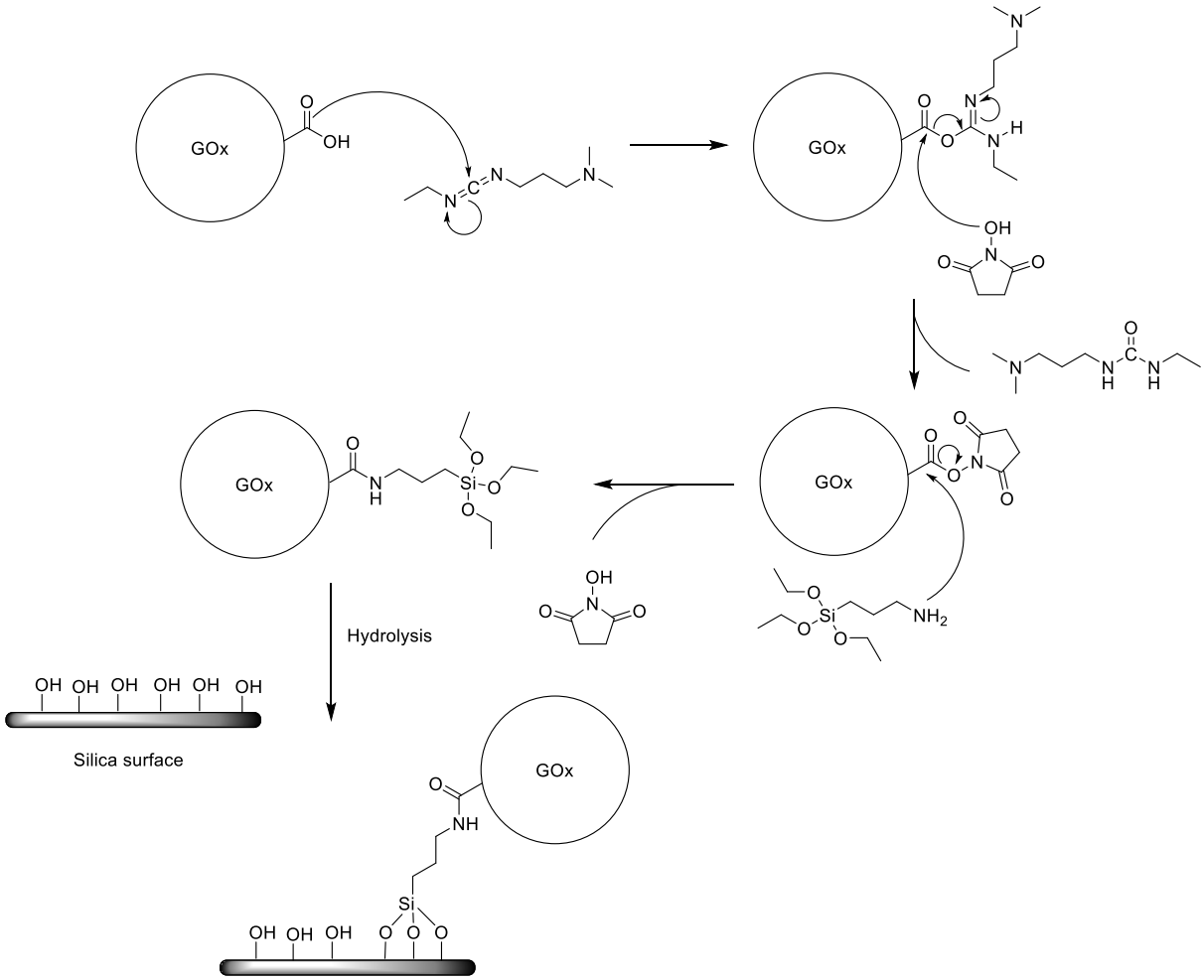
For the quantification of intracellular GSH level, MDA-MB-231 breast cancer cells were seeded at a density of 2×10^5 cells per well into a 6-well glass plate and incubated for 24h in DMEM high glucose medium (2mL) with 10% FBS and 1% penicillin at 37 °C with 5% CO_2 humidified atmosphere. After the treatment, the cells were incubated with ThiolTracker™ Violet dye working solution (20 μM) for 30 min and washed three times with PBS for flow cytometry measurements. ThiolTracker™ Violet was excited at 405 nm, and fluorescence was detected from 500 to 600 nm. Data were analyzed with Flowjo software.

Flow cytometric apoptosis and flow uptake studies

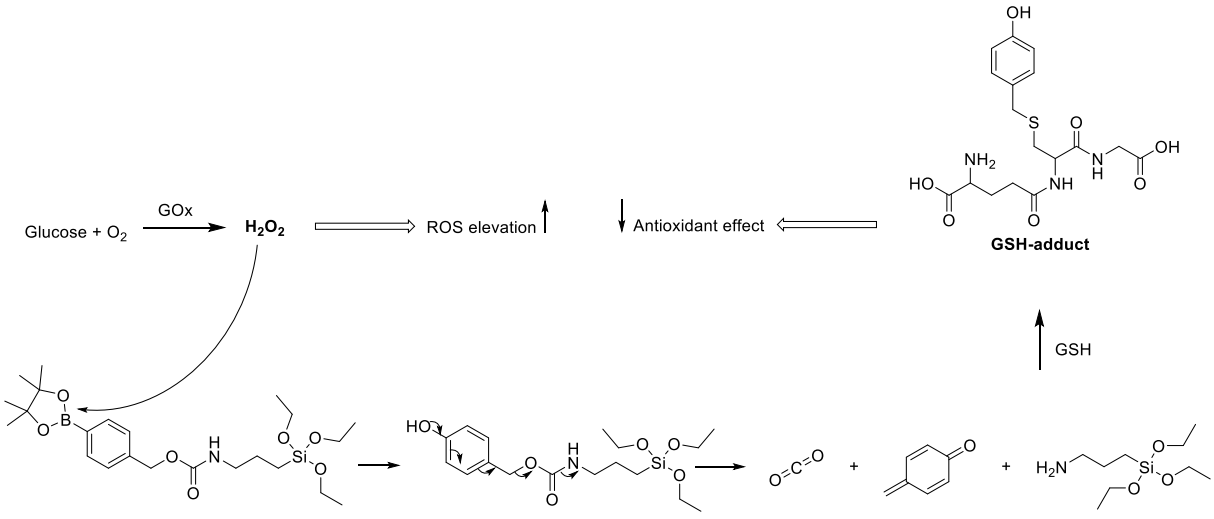
For the flow cytometric apoptosis study, cancer cells were seeded in 6-well plates at a density of 2×10^5 cells per well in DMEM (2 mL) containing 10% FBS and 1% penicillin at 37 °C in 5% CO_2 humidified atmosphere. After 24 h incubation, the medium was replaced with fresh one (1.7 mL) and doped with glucose (0.1mL, 20 mg/mL), followed by the addition of free GOx, ORMOSIL or ORMOSIL@GOx. After 24h incubation, cells in each well were collected in a 2mL eppendorf tube and washed twice with PBS buffer solution. The cells were washed twice with PBS and treated with trypsin (0.5 mL) for 2 min. Subsequently, the cells were re-suspended in fresh medium (0.5 mL) and collected by centrifugation at 3500 rpm for 3 min. After one more round of washing with PBS (0.5 mL) and centrifugation, the cells were re-suspended in 1× Annexin V binding buffer (100 μL), followed by the addition of FITC annexin V (5 μL) and PI working solution (1 μL, 100 μg/mL). After incubation at room temperature for 15 min, 1× Annexin V binding buffer (400 μL) was added and the samples were subjected to the flow cytometry analysis.

Cellular uptake study

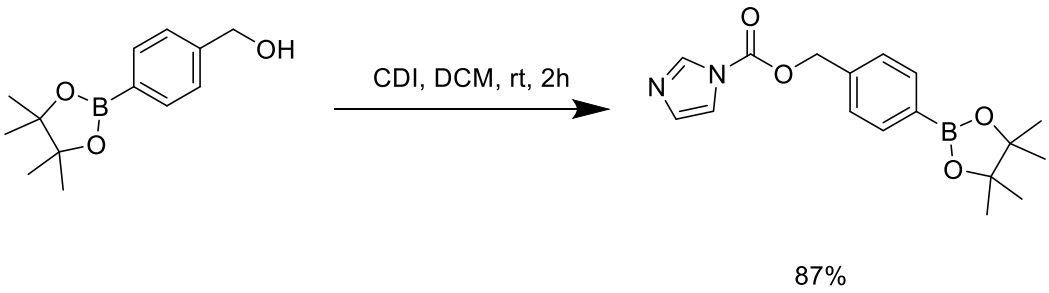
MDA-MB-231 breast cancer cells were seeded on glass cover slip in 6-well plates and allowed to grow for 24 h. The cells were then incubated with cell culture medium containing FITC-labeled GOx and ORMOSIL@GOx (250 mU mL^{-1}) for 2h. After which, the cells were washed with PBS three times, and LysoTracker™ Deep Red, followed by Hochest 33342 were used to stain the nucleus. After the treatment, cells were imaged by CLSM (ZEISS LSM 800). Fluorescence was examined under excitation at 405 nm for Hochest 33342, 488 nm for FITC, and 630 nm for LysoTracker™ Deep Red.



Scheme S1. Schematic illustration of the conjugation mechanism between glucose oxidase and silica nanoparticles. Briefly, the –COOH groups of glucose oxidase conjugate with the –NH_2 group of Si-PE. Then, the –$Si(OEt)_3$ groups of the modified glucose oxidase could conjugate with the silica nanoparticles *via* silica hydrolysis.

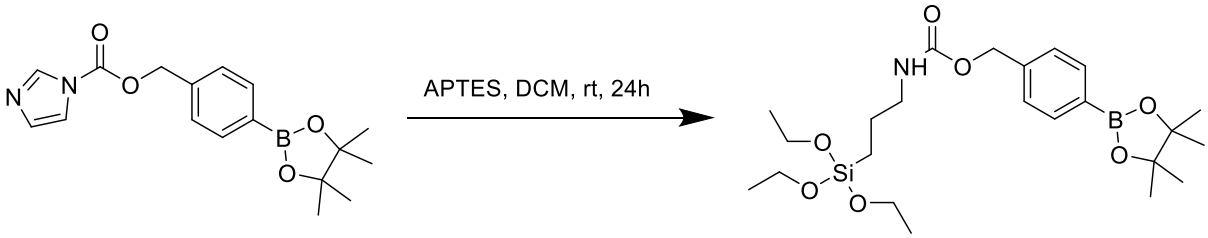


Scheme S2. Schematic illustration for catalytic and therapeutic mechanism with ORMOSIL@GOx.



87%

Scheme S3. Schematic illustration for the synthesis of CDI-PE.



50%

Scheme S4. Schematic illustration for the synthesis of Si-PE.

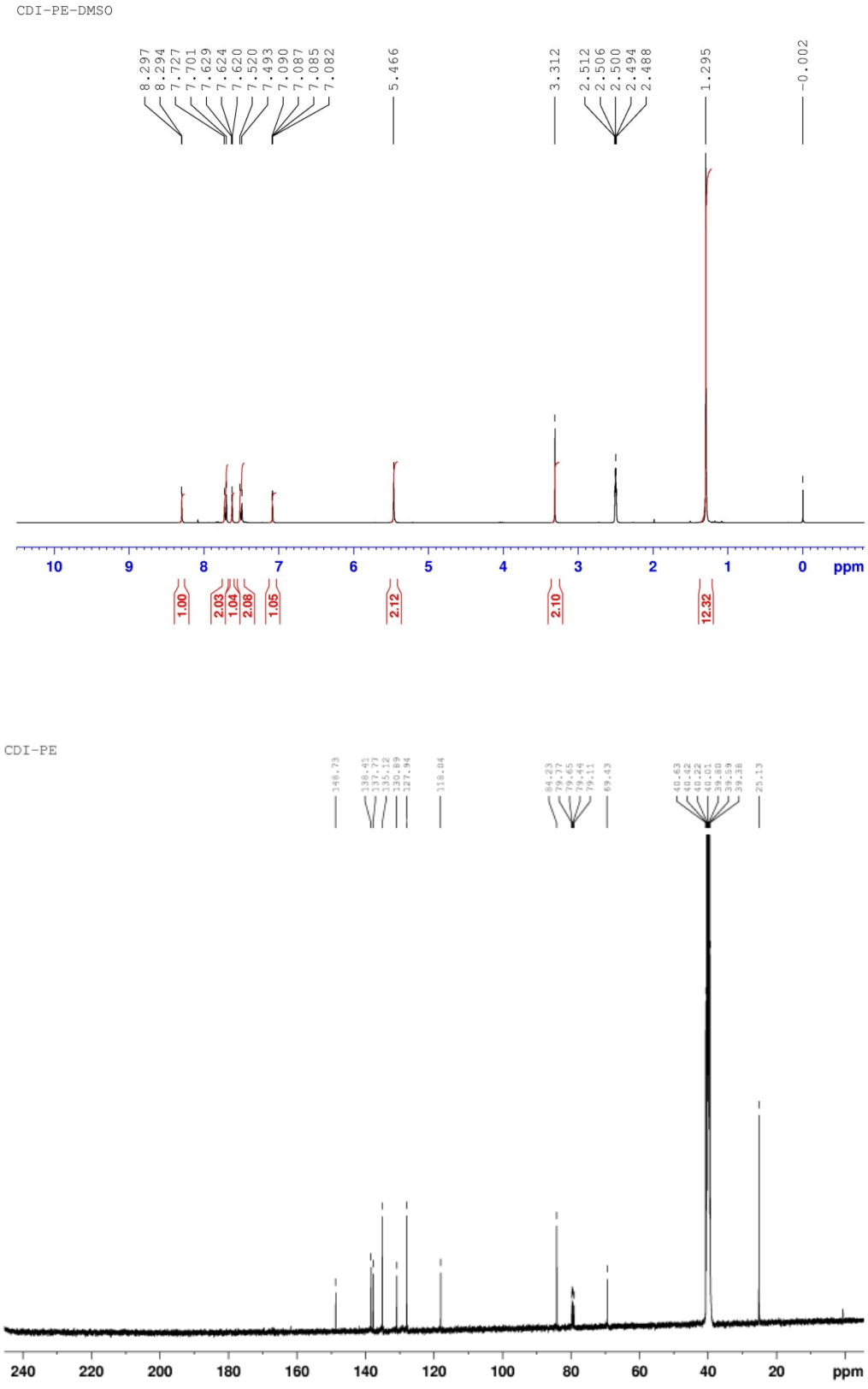
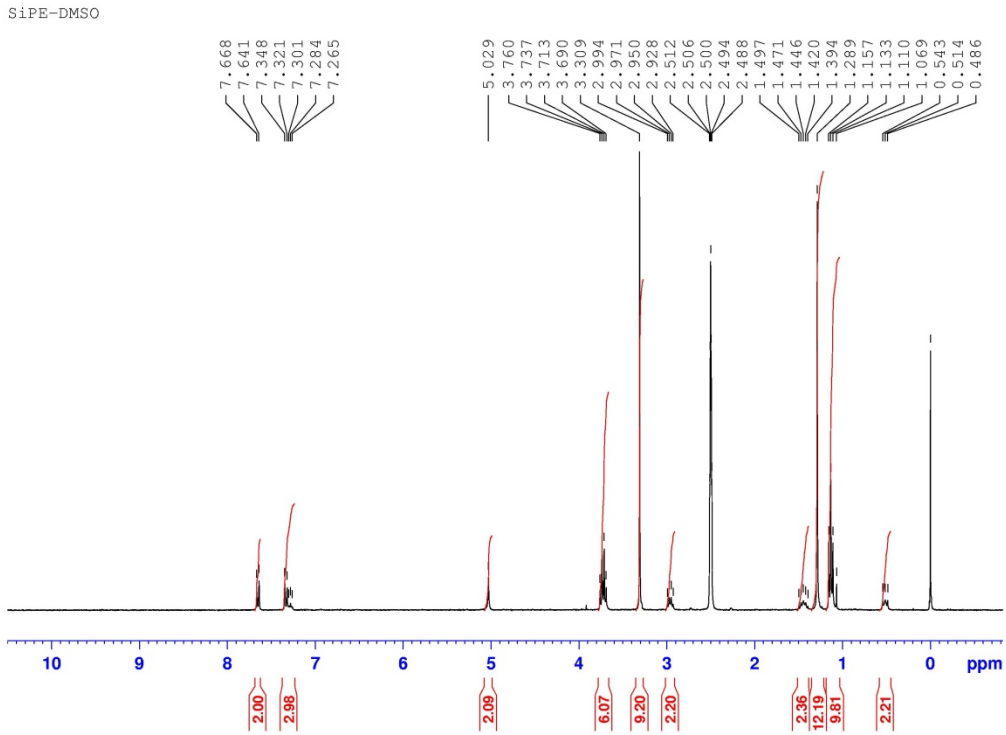
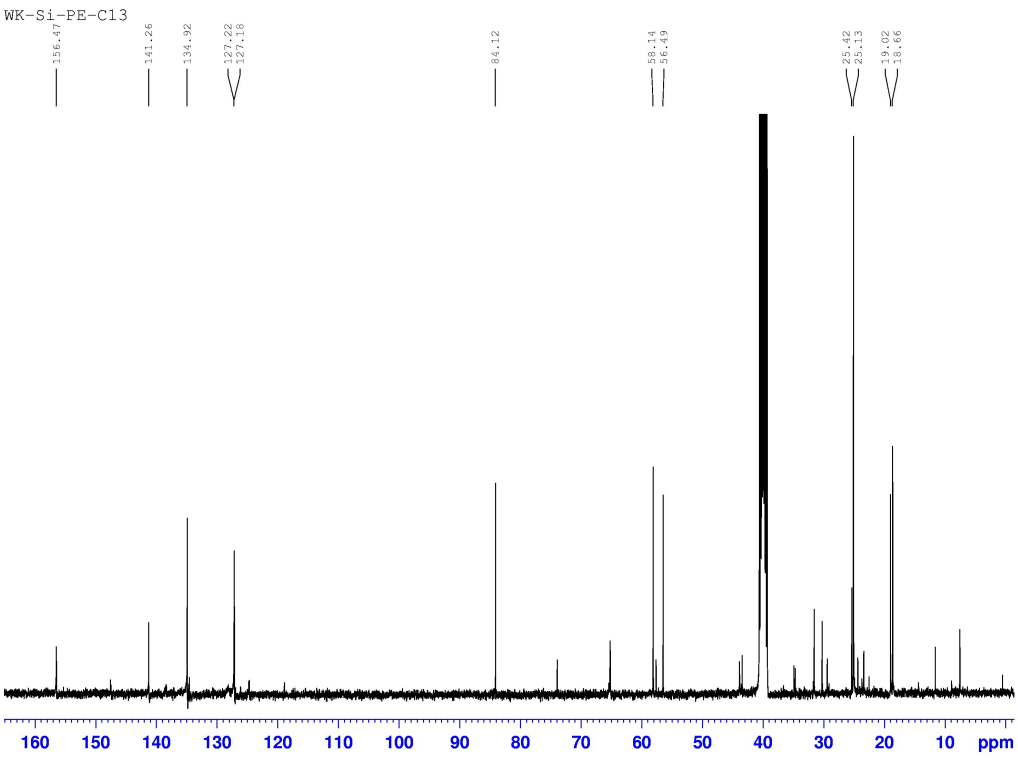


Figure S1. ^{1}H NMR (top) and ^{13}C NMR (bottom) spectra of CDI-PE.


SiPE-DMSO
7.668
7.641
7.348
7.321
7.301
7.284
7.265
5.029
3.760
3.737
3.713
3.690
3.309
2.994
2.971
2.950
2.928
2.512
2.506
2.500
2.494
2.488
1.497
1.471
1.446
1.420
1.394
1.289
1.157
1.133
1.110
1.069
0.543
0.514
0.486
10 9 8 7 6 5 4 3 2 1 0 ppm
2.00
2.98
2.09
6.07
9.20
2.20
2.36
12.19
9.81
2.21


WK-Si-PE-C13
156.47
141.26
134.92
127.22
127.18
84.12
58.14
56.49
25.42
25.13
19.02
18.66
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

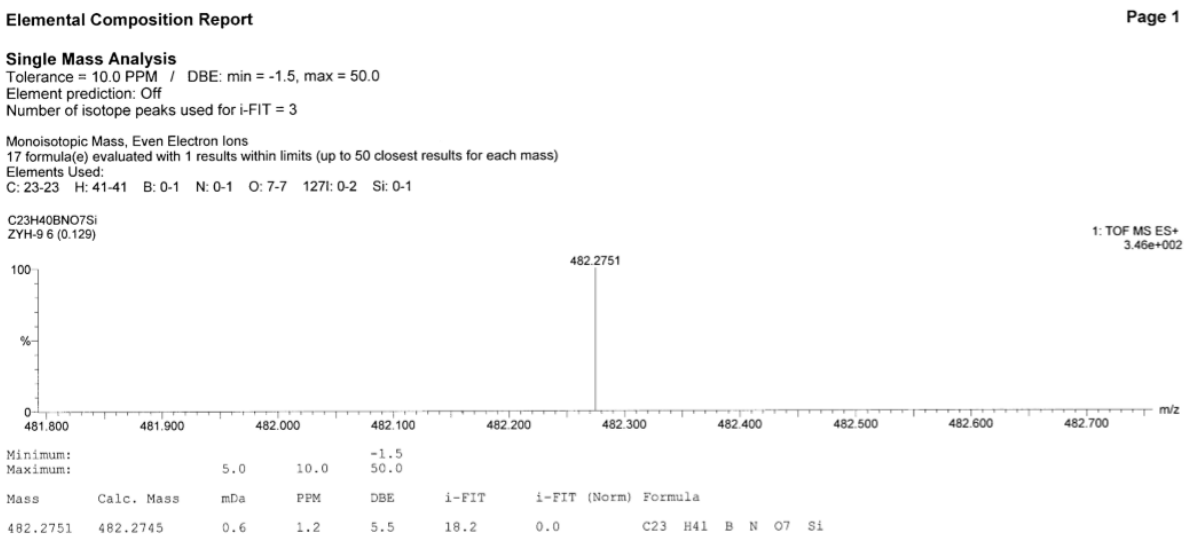


Figure S2. ^{1}H NMR (top), ^{13}C NMR (middle), and HRMS (bottom) spectra of Si-PE.

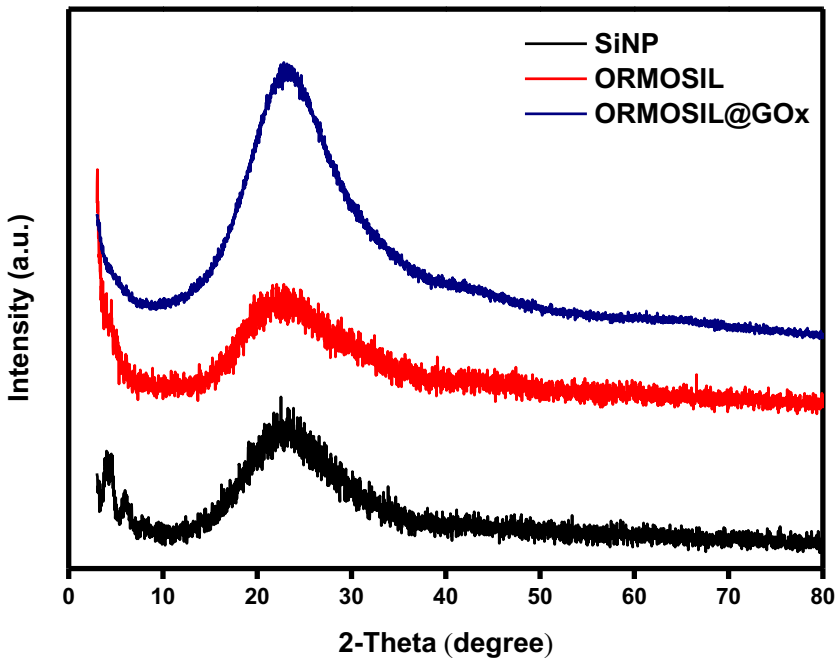


Figure S3. Powder XRD analysis for SiNP, ORMOSIL and ORMOSIL@GOx.

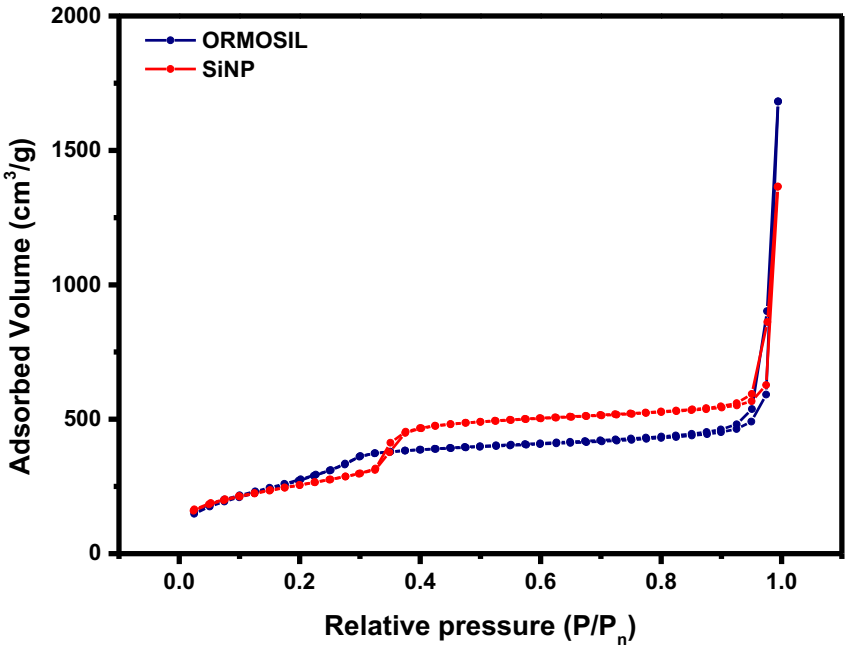


Figure S4. BET isotherm patterns for SiNP and ORMOSIL.

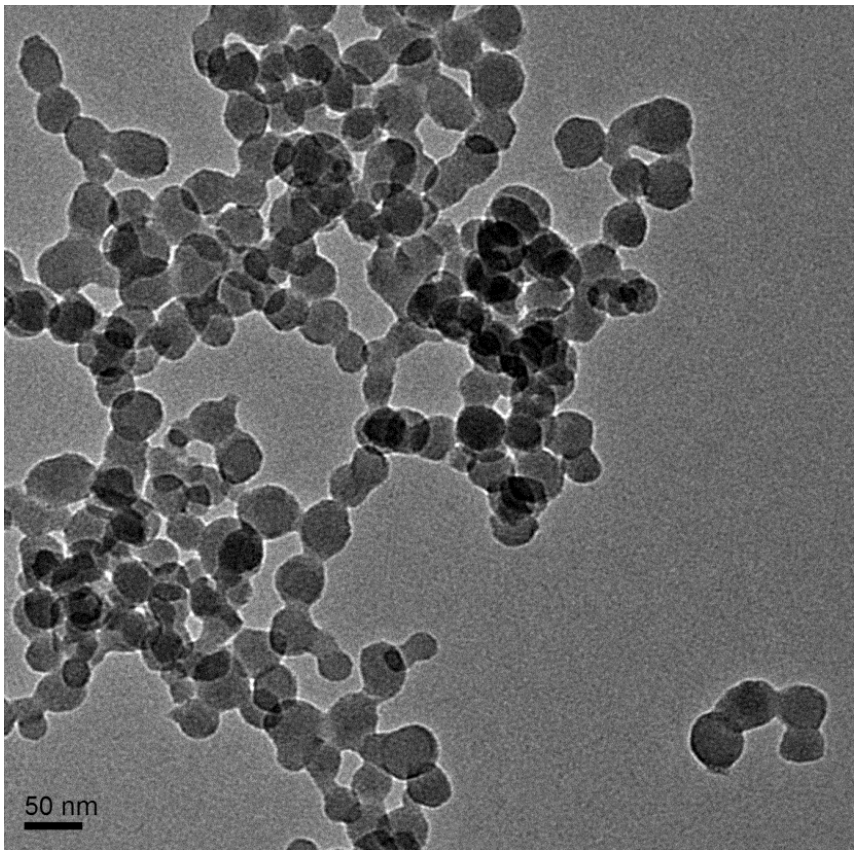


Figure S5. TEM image of ORMOSIL.

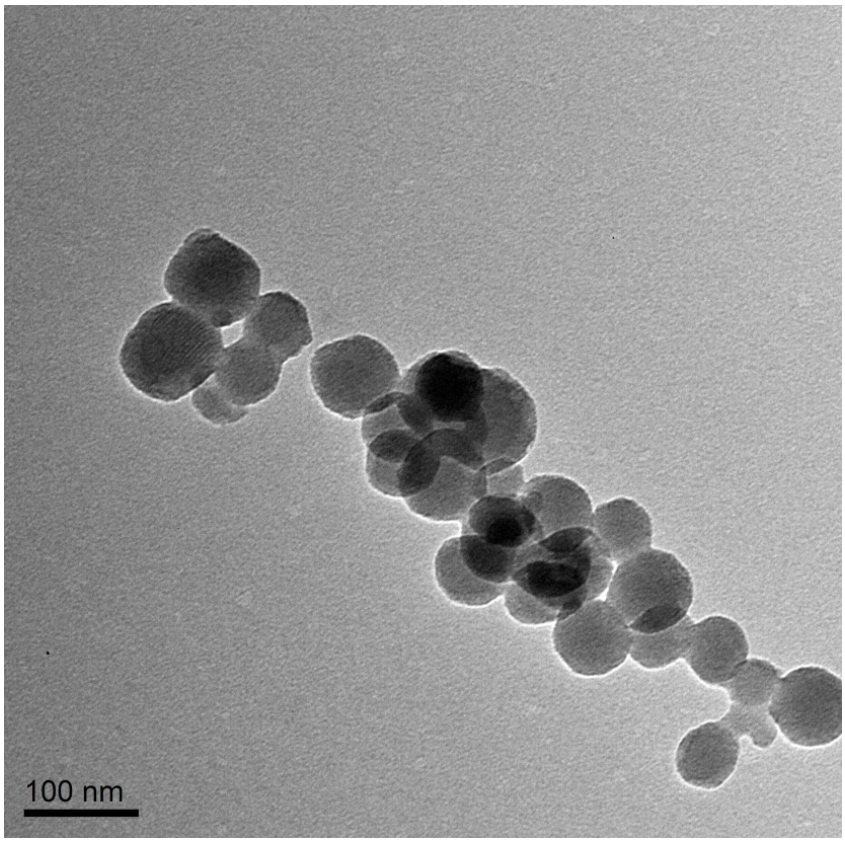


Figure S6. TEM image of ORMOSIL@GOx.

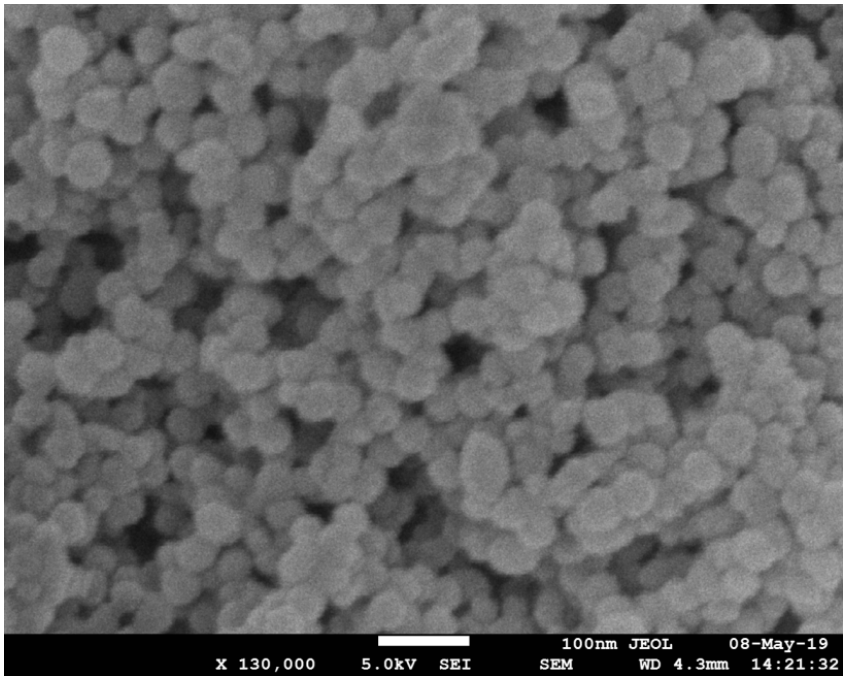


Figure S7. SEM image of ORMOSIL@GOx.

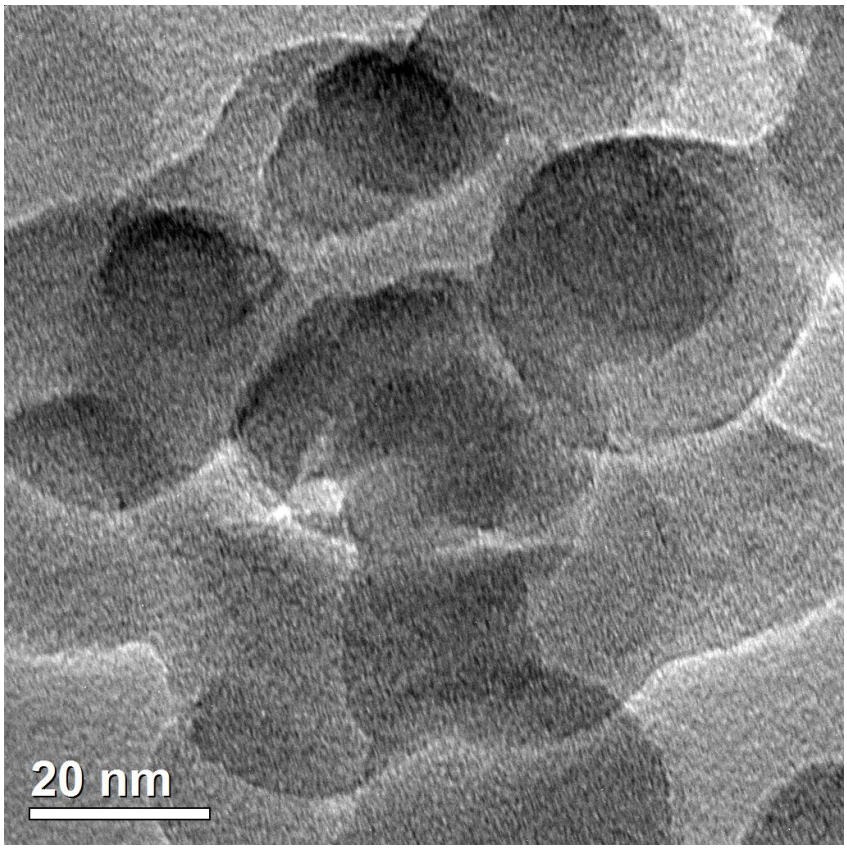


Figure S8. Highly magnified TEM image of ORMOSIL.

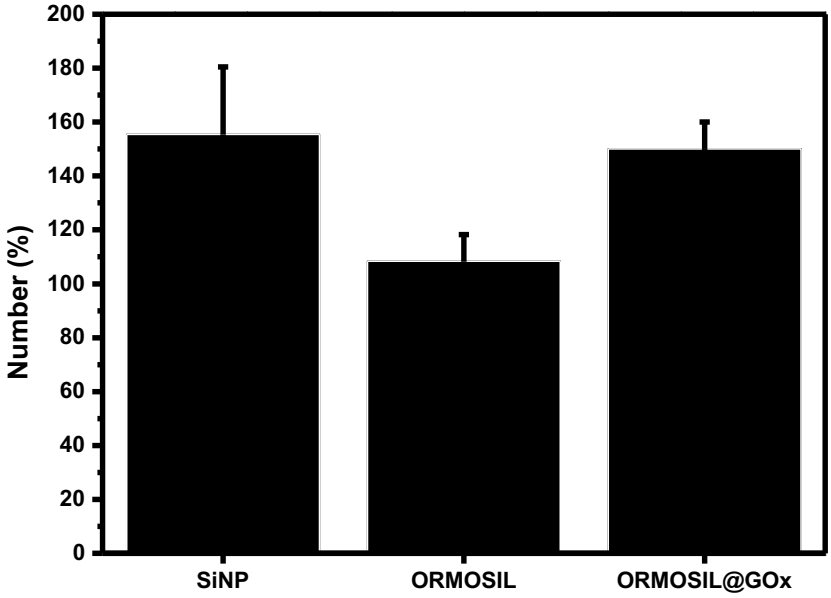


Figure S9. Hydrodynamic size distribution of SiNP, ORMOSIL and ORMOSIL@GOx as measured by dynamic light scattering (DLS).

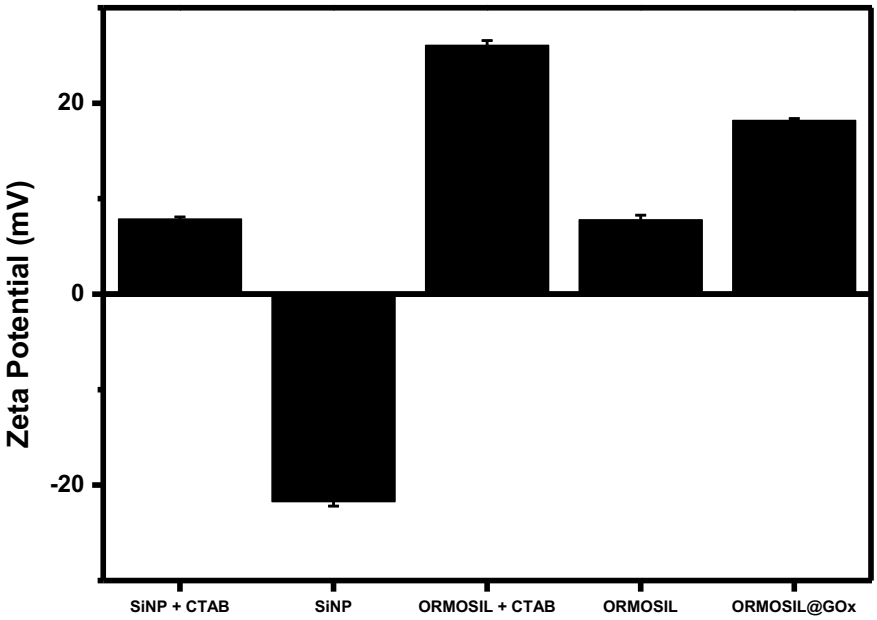


Figure S10. Zeta potential change of SiNP, ORMOSIL and ORMOSIL@GOx.

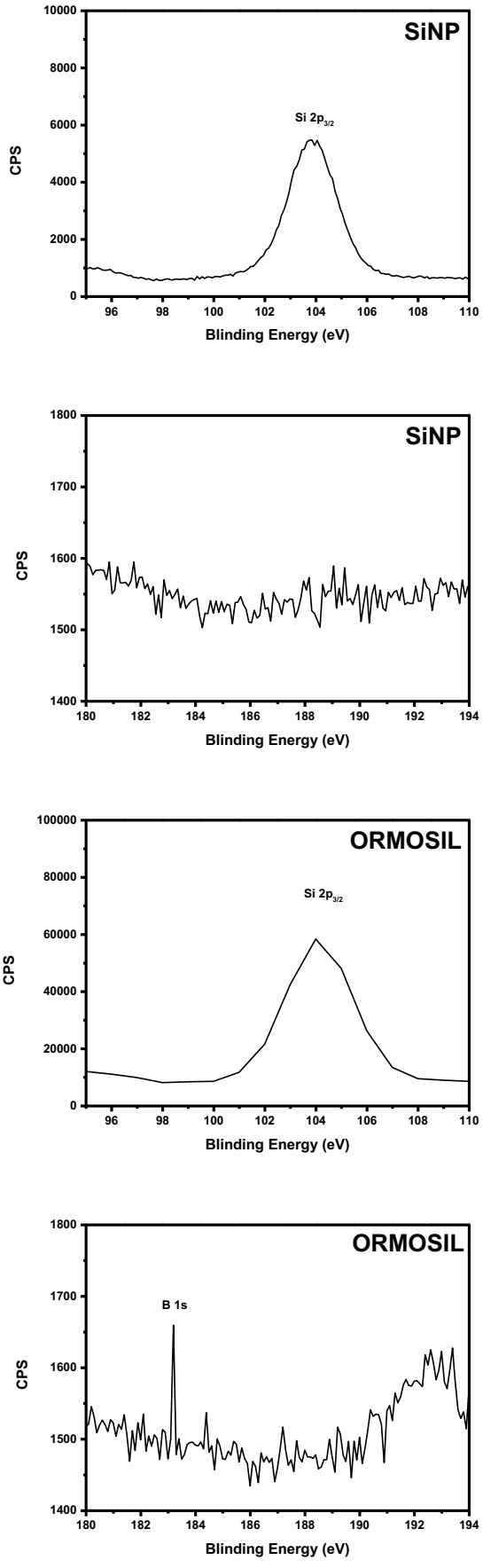


Figure S11. XPS spectra of SiNP and ORMOSIL.

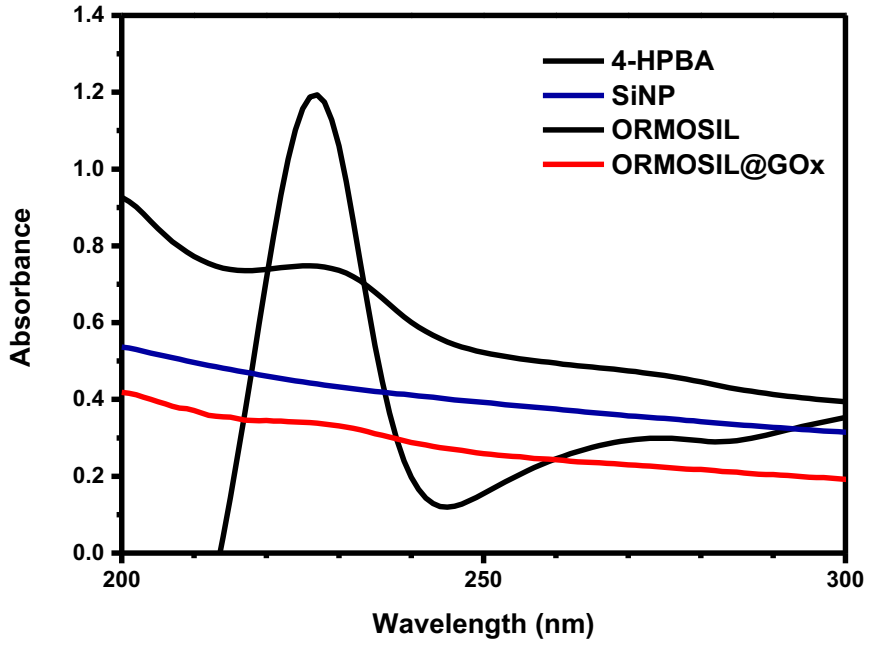


Figure S12. UV-Vis absorbance showing the absence and presence of 4-HPBA in SiNP, ORMOSIL and ORMOSIL@GOx.

$$\text{Molar F/P} = \frac{\text{MW}}{389} \times \frac{A_{495} / 195}{A_{280} - (0.35 \times A_{495}) / E^{0.1\%}}$$

$$= \frac{A_{495} \times C}{A_{280} - (0.35 \times A_{495})}$$

$$\text{where } C = \frac{\text{MW} \times E^{0.1\%}{}_{280}}{389 \times 195}$$

Molar F/P = **4.197160726**

C is a constant value given for a protein.
MW is the molecular weight of the protein.
389 is the molecular weight of FITC.
195 is the absorption $E^{0.1\%}$ of bound FITC at 490 nm at pH 13.0.
$(0.35 \times A_{495})$ is the correction factor due to the absorbance of FITC at 280 nm.
$E^{0.1\%}$ is the absorption at 280 nm of a protein at 1.0 mg/ml.
A_{495} is the absorption at 495 nm of a conjugated protein.
A_{280} is the absorption at 280 nm of a conjugated protein.

Figure S13. Calculation formula for the number of FITC conjugated to GOx.

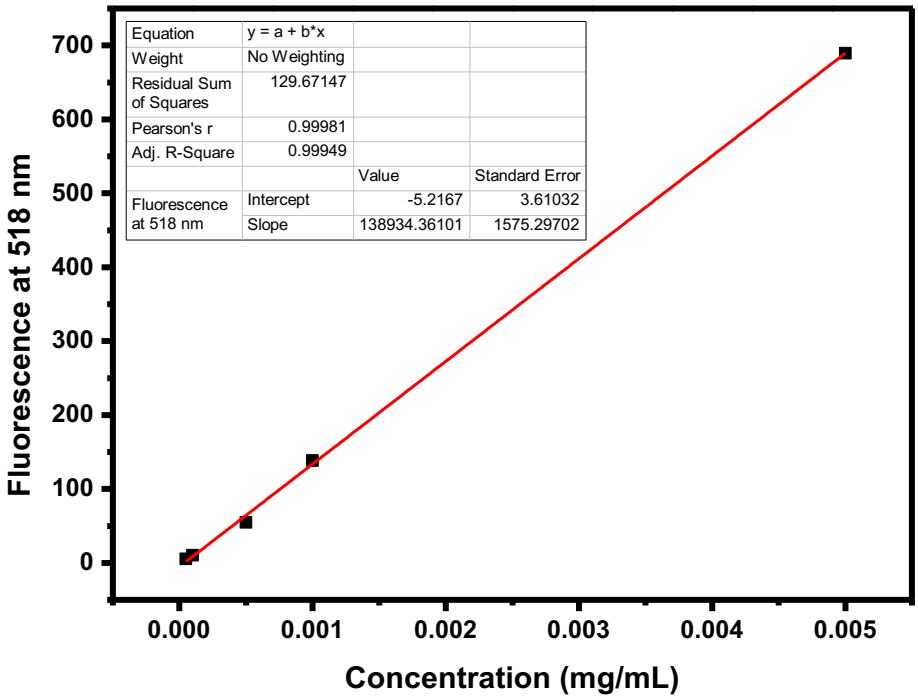
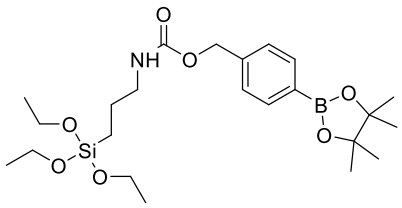


Figure S14. Calibration curve for the determination of FITC-labeled GOx.



Chemical Formula: $C_{23}H_{40}BNO_7Si$
Molecular Weight: 481.47

Total mass of silane ligand added = 130mg
Total mass of TEOS added = 0.6mL × 0.94 g mL^{-1} = 564mg
Assuming total incorporation of silane ligand, %wt. content of silane ligand = 18.7%
Actual %wt. content of silane ligand = 11%
Loading efficiency = **59%**

Figure S15. Loading efficiency of the silane ligand in ORMOSIL.

Table S1. Michael-Menten constant (K_m) and maximum velocity (V_{max}) calculation and plot points for ORMOSIL@GOx using microplate reader data collected from hydrogen peroxide assay.

<>	1	2	3	4	5
F	0.3481	0.3717	0.4528	0.4989	0.6854
G	0.3503	0.3743	0.4240	0.5430	0.6199
H	0.3597	0.3709	0.4038	0.5054	0.5408
End Time:	5/28/2019 8:21:16 PM				
	0.352700	0.372300	0.426867	0.515767	0.615367
	0.006161	0.001778	0.024625	0.023808	0.072407
	0.003557	0.001026	0.014218	0.013745	0.041804
	1.008548	0.275670	3.330669	2.665030	6.793337
Calibration equation for H_2O_2	y = 8.3712x + 0.3314				
Glucose concentration (mM)	0.5	1	2	5	10
H_2O_2 produced (mM)	0.002544	0.004886	0.011404	0.022024	0.033922
Time until end of exp. (s)	1800	1800	1800	1800	1800
H_2O_2 produced per sec. (mM s^{-1})	1.41E-06	2.71E-06	6.34E-06	1.22E-05	1.88E-05
H_2O_2 produced per sec. (M s^{-1})	1.41E-09	2.71E-09	6.34E-09	1.22E-08	1.88E-08
H_2O_2 produced per sec. (x10^{-8} M s^{-1})	0.141358	0.271433	0.633566	1.223551	1.884548
(Glucose conc.)$^{-1}$ (mM^{-1})	2	1	0.5	0.2	0.1
V^{-1} (x10^8) (M^{-1} s)	7.074252	3.684147	1.578369	0.817293	0.530631
$1/V_{max}$ from Lineweaver-Burk plot	0.0782				
V_{max}	12.7877 (x10^{-8} M s^{-1})				
K_m/V_{max} from Lineweaver-Burk plot	3.4983				
K_m	44.7 mM				

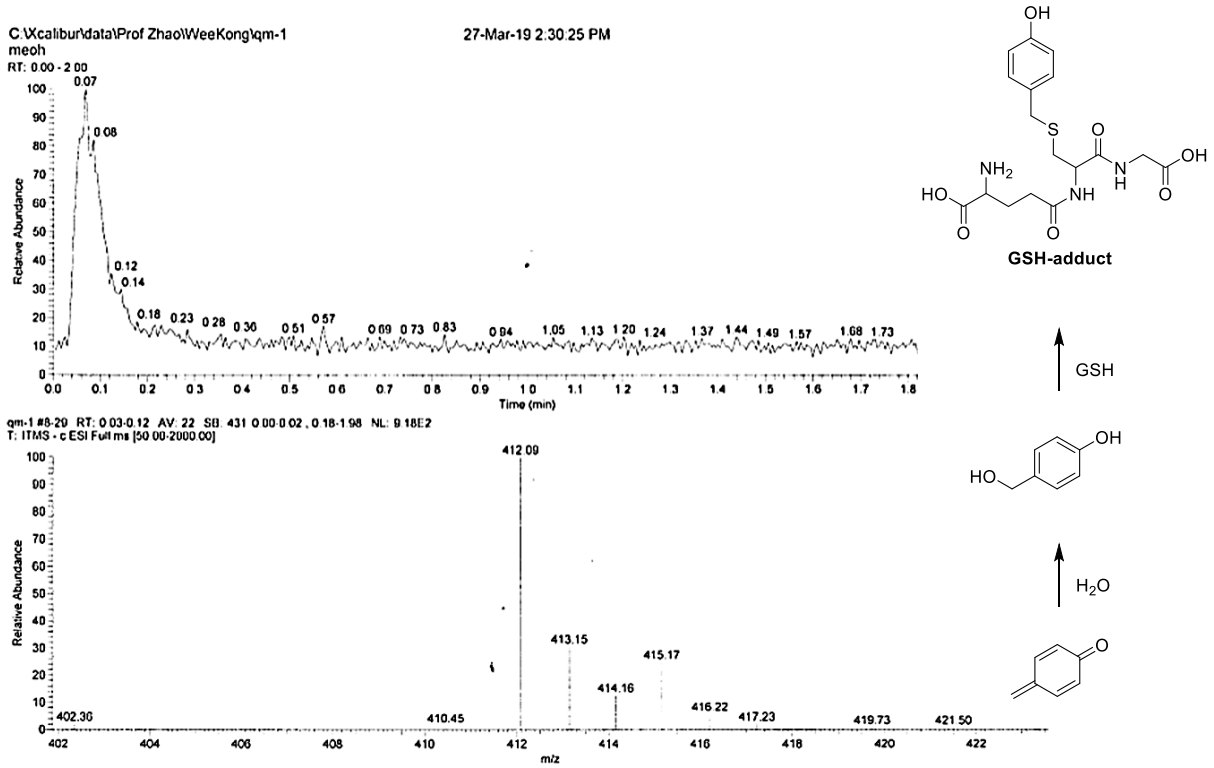


Figure S16. LCMS analysis of ORMOSIL@GOx incubated in 1mM glucose and 1mM GSH over 24h.

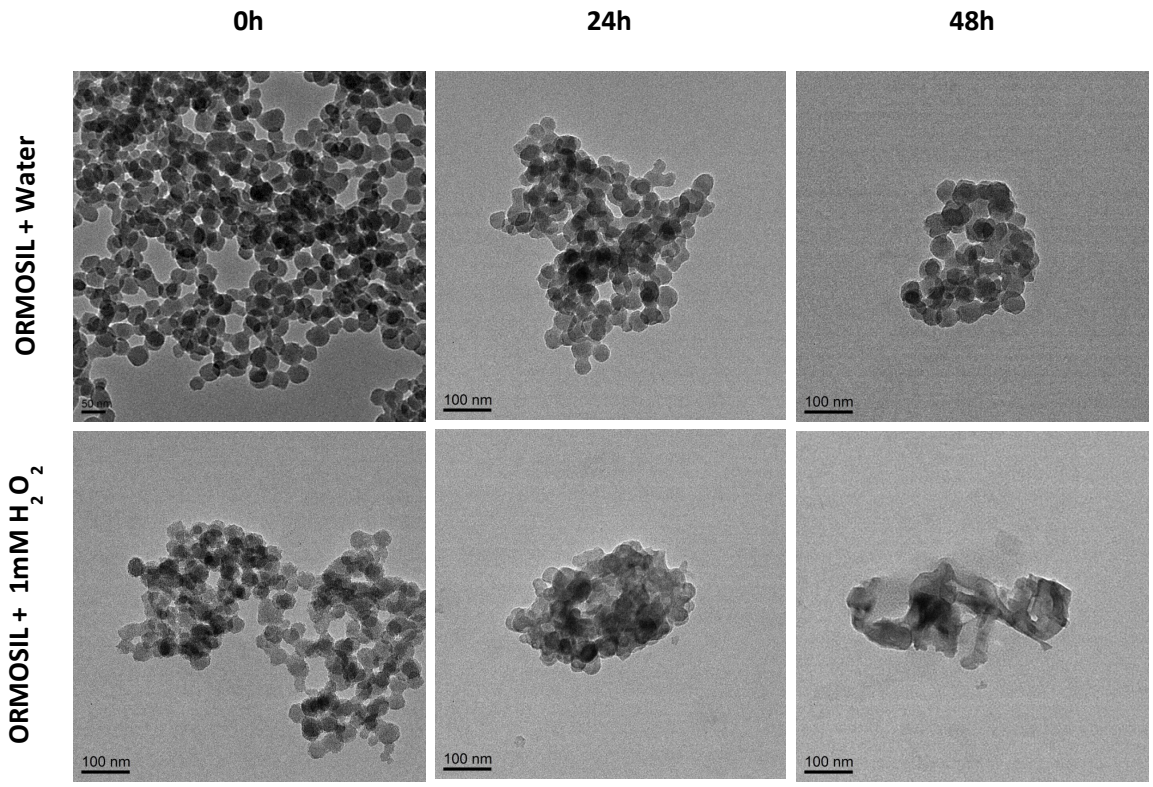


Figure S17. TEM images of ORMOSIL in H_2O and H_2O_2 over 48h.

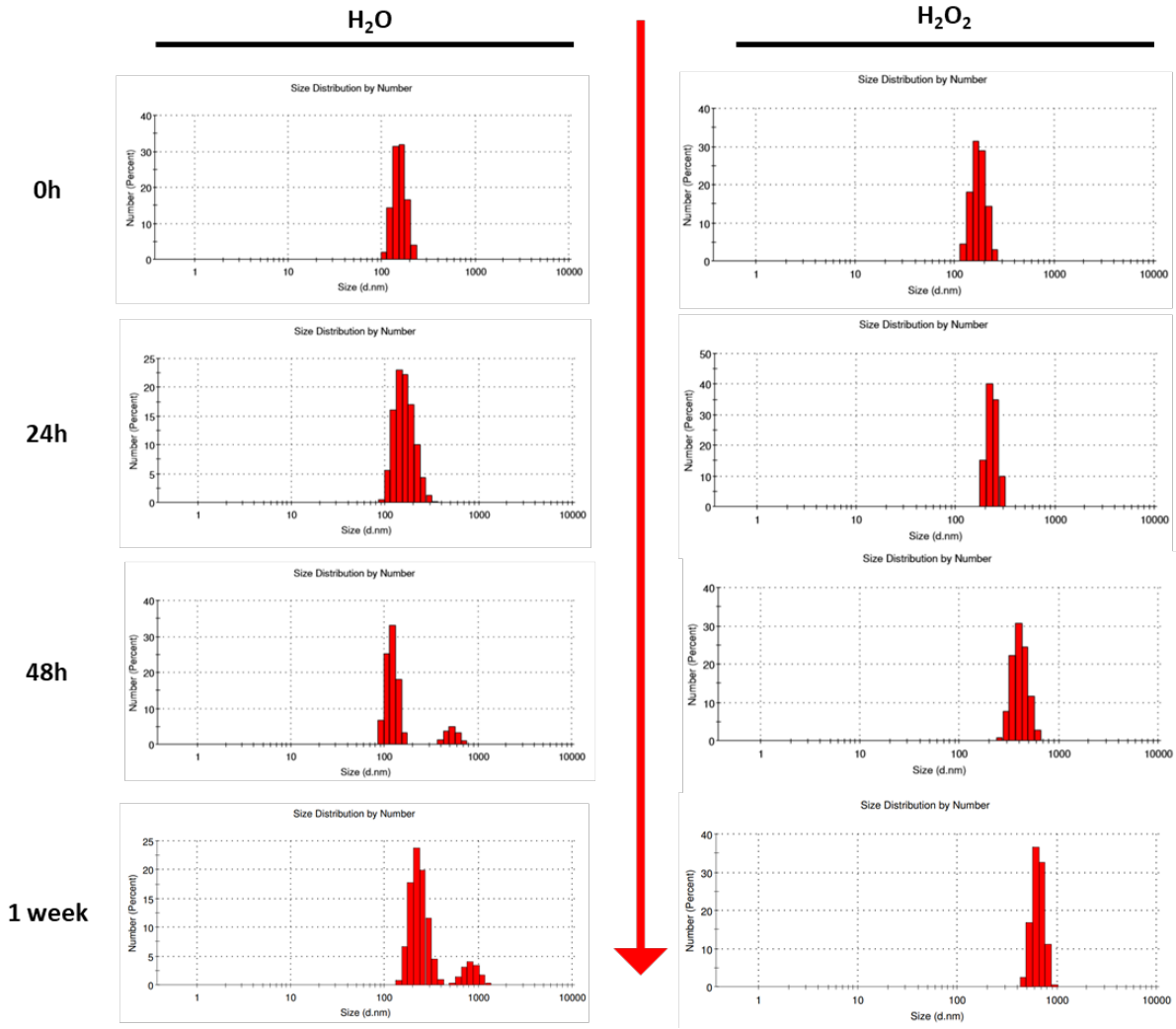


Figure S18. DLS comparison of ORMOSIL in H_2O and H_2O_2 over 1 week.

The silane ligand in ORMOSIL could break down when subjected to a high level of hydrogen peroxide over time. Hydrogen peroxide produced from the catalysis of glucose allows a cascade reaction to cleave the C–O bond of pinacol ester. Since the content of the silane ligand in ORMOSIL is significant, the morphological change upon the treatment was investigated. ORMOSIL was incubated in both deionized water and 1 mM H_2O_2 aqueous solution over three days and monitored by TEM and DLS. It was observed that the spherical shape was intact in aqueous medium over 48 h. In the H_2O_2 system, however, the nanoparticles aggregated after 24 h and eventually merged into a lump after 48 h (Fig. S17, ESI). This is due to the loss of stability due to the chemical change. DLS comparison between the two again confirmed that the H_2O_2 system was significantly more aggregated at each time point (Fig. S18, ESI). A greater difference was found after 1 week of study.

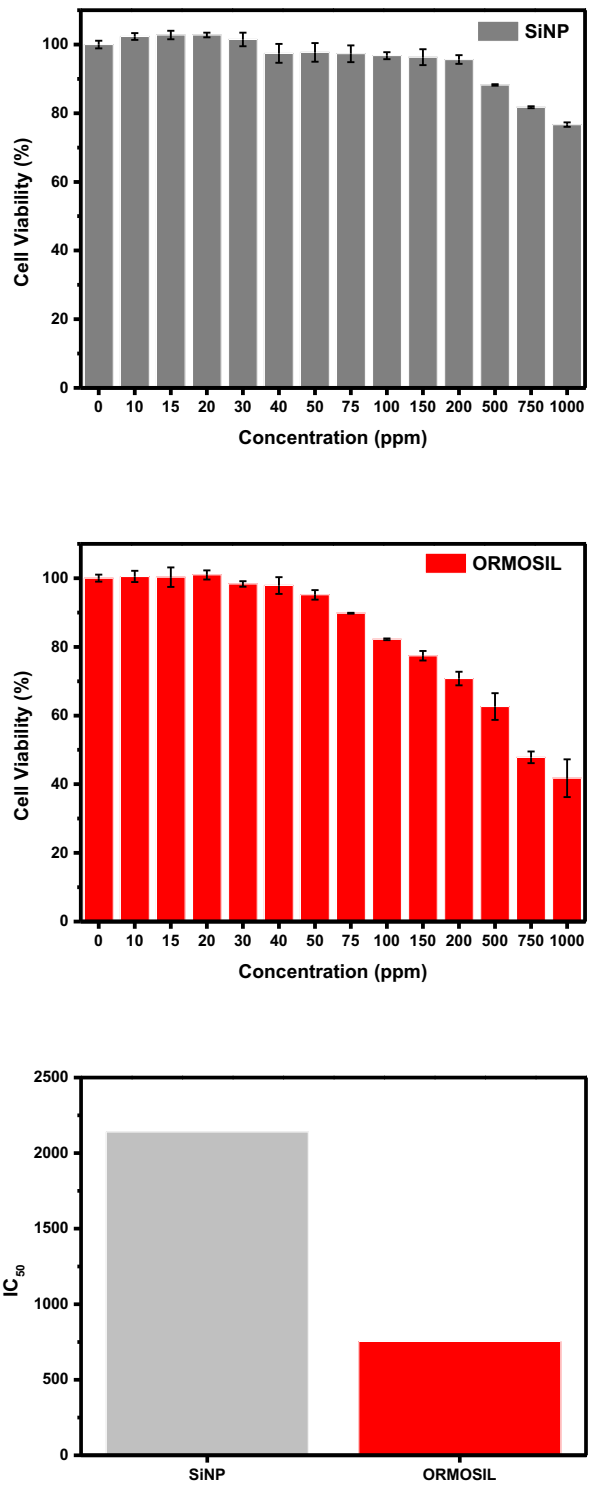


Figure S19. IC_{50} determination of SiNP and ORMOSIL MDA-MB-231 breast cancer cells.

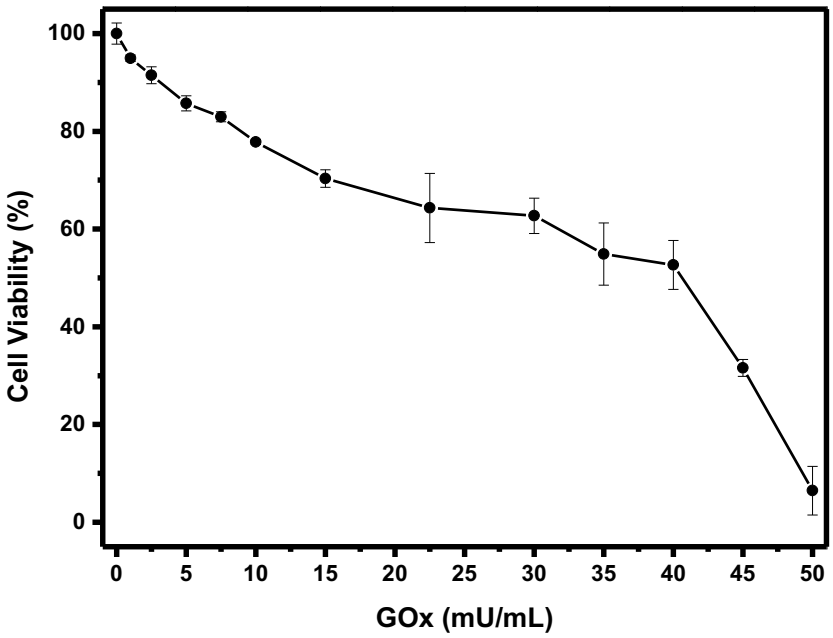


Figure S20. IC_{50} determination of the free GOx in MDA-MB-231 breast cancer cells.

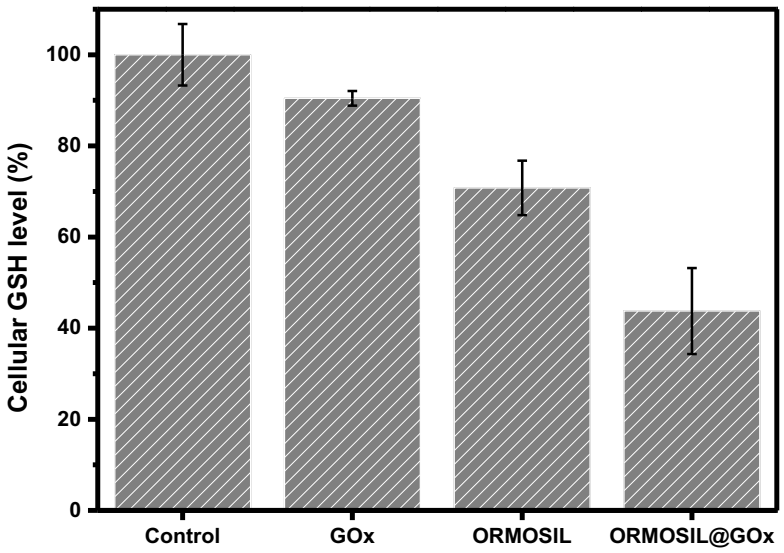


Figure S21. Level of glutathione depleted by the nanosystems without additional glucose.

Table S2. Rate of hydrogen peroxide scavenging by the nanosystem. The rate of hydrogen peroxide scavenging was determined to be 73.8 mM g^{-1} h^{-1}. Briefly, the nanosystem (100 μg/mL) was incubated in 0.196 mM hydrogen peroxide in a microplate over a period of 24h. After which, pierce hydrogen peroxide kit was used to evaluate the fluorescence at 595nm and hydrogen peroxide concentration remained in the nanosystem and the blank control.

<>	11	12	
A	0.6851	0.3408	
B	0.5682	0.3472	
C	0.6758	0.3517	
End Time:	9/17/2019 9:36:38 PM		
Average	0.643033	0.346567	
Raw SD	0.064974	0.005478	
SD/SQRT	0.037513	0.003162	
Final SD%	5.833736	0.912511	
	Control with only H_2O_2	H_2O_2 with 100μg/mL nanosystem	
mM	0.037227	0.001812	Using y = 8.3712x + 0.3314
	Change in H_2O_2		
H_2O_2 Conc. Δ	0.035415	mM	
Time Δ	24	hours	
NP conc.	100	μg/mL	
NP mass	0.00002	g	
H_2O_2 scavenging rate	73.78141	mM g^{-1} h^{-1}	

References

1. W. Fan, N. Lu, P. Huang, Y. Liu, Z. Yang, S. Wang, G. Yu, Y. Liu, J. Hu, Q. He, J. Qu, T. Wang and X. Chen, *Angew. Chem. Int. Ed.*, 2017, **56**, 1229-1233.
2. C. Pisani, J.-C. Gaillard, M. Odorico, J. L. Nyalosaso, C. Charnay, Y. Guari, J. Chopineau, J.-M. Devoisselle, J. Armengaud and O. Prat, *Nanoscale*, 2017, **9**, 1840-1851.
3. J. Li, A. Dirisala, Z. Ge, Y. Wang, W. Yin, W. Ke, K. Toh, J. Xie, Y. Matsumoto, Y. Anraku, K. Osada and K. Kataoka, *Angew. Chem. Int. Ed.*, 2017, **56**, 14025-14030.