Supporting Information

SERS monitoring of local pH in encapsulated therapeutic cells

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General Experimental Procedures

Materials

Hexadecyltrimethylammonium chloride (CTAC, 25% W/W), L-ascorbic acid (≥99%), sodium hypochlorite solution (10−15% available chlorine), sodium borohydride ($NaBH_4$, 99%), silver nitrate ($AgNO_3$, ≥99%), sodium citrate tribasic dihydrate (≥98%), O-[2-(3-mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol (PEG-SH, MW=5000 g/mol), 4-mercaptobenzoic acid (4MBA, 99%), sodium hydroxide, poly(allylamine hydrochloride) (PAH) average Mw = 15 000), hydrogen peroxide (H_2O_2, 28%), sulfuric acid (H_2SO_4, 98%), ethanol (EtOH, 99.8%) and poly-L-Lysine hydrobromide (PLL, 15-30 kDa) were supplied by Sigma – Aldrich. Hydrochloric acid (37 wt %) was purchased from Panreac. Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$, ≥99.9%) from Alfa Aesar was employed without further purification. Milli-Q grade water was used in all experiments. Ultra-pure low-viscosity (20-200 mPa*s) and high guluronic (LVG) acid alginate (G/M ratio > 1.5) with a MW of 75-200 kDa was purchased from FMC Biopolymer (Norway).

Synthesis of SERS-active pH sensor

AuNSs were synthesized following a previously reported seed-mediated growth method,[1] aiming at a plasmon resonance around 780 nm. Briefly, gold seeds were prepared by the classic Turkevich method.[2] For tip growth, 100 μL of 125 mM $HAuCl_4$ solution, 50 μL of 1 M HCl, and 2.5 mL of seed solution were sequentially added into 50 mL of nanopure water under vigorous stirring. Subsequently, 500 μL of 3 mM $AgNO_3$ and 250 μL of 100 mM AA were quickly added at the same time. A rapid color change from red to dark blue indicates AuNSs formation. Subsequently, 410 μL of 0.1 mM PEG-SH was added into the resulting solution and stirred for 10 min. After washing by centrifugation at 1190 g for 15 min, AuNSs were redispersed in water and the gold concentration was adjusted to 0.5 mM. For functionalization with the Raman reporter we followed a previously reported protocol.[3] 1 mL of 0.05 mM 4MBA solution was added dropwise to 5 mL of as-synthesized AuNSs colloid under mild stirring for 1 hour. To further stabilize the nanoparticles, 200 μL of 0.1 mM PEG-SH was added and stirred for 15 min. The resulting nanoparticles were washed

twice by centrifugation at 580 g for 10 min and redispersed in water. Negatively charged, MBA-coated AuNSs were additionally functionalized with PAH polyelectrolyte by adding 0.5 mL of AuNSs ([Au] = 0.3 mM) dropwise into a PAH solution (0.5 mL of 2 mg/mL). After incubating for 30 min in the dark with gentle shaking, the solution was centrifuged for 10 min at 1150 g. The supernatant was then removed, and the blue pellet was re-suspended in water and centrifuged again for 10 min at 6000 rpm. Size, z-potential and optical properties of the prepared SERS-active pH sensor were characterized by transmission electron microscopy (TEM), electrophoretic mobility and UV-Vis spectroscopy, respectively (Figures 1 and S1).

Preparation of microencapsulated cells

Murine C2C12 myoblasts were grown in T-flasks with Dulbecco´s modified Eagles´s medium supplemented with inactivated 10% FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic solution, at 37 °C in a 5% CO2/ 95% air atmosphere, and passaged every 2−3 days. All reagents were purchased from Gibco (Life Technologies, Spain).

Alginate 1.5% (FMC Biopolymer, Norway) without cells or with cells suspended in 1.5% alginate at a density of 5x106 cells/mL, was extruded in an electrostatic atomization generator (Nisco®, Switzerland) and the resulting microcapsules were completely gelled by shaking for 10 min in a 55 mM $CaCl_2$ solution. Next, microcapsules were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a second coating with 0.1% alginate for another 5 min. The whole procedure was performed at room temperature, under aseptic conditions and using complete medium. The morphology and diameter of the microcapsules were assessed using an inverted optical microscope (Nikon TSM, Japan).

SERS labelling of capsules

To label alginate capsules for SERS, 300 µL of microcapsules was rinsed with 200 µL NaCl solution 0.9% (w/v). The saline solution was then thoroughly removed, and the SERS-active pH sensor solution was added at a density of 0.5-2 AuNSs/µm2. The Eppendorf tube was sealed, taped horizontally onto a platform rocker and stirred for 5 min, at medium speed. The capsules were rinsed again with NaCl solution 0.9% (w/v) and

placed in complete cell culture media (DMEM supplemented with 10% FBS) (400 μL) for SERS measurements.

Protocol for pH Calibration in Capsule

The pH calibration curve was determined by measuring probe-embedded capsules in complete cell culture media (DMEM supplemented with 10% FBS), with a pH within the range from 3.7 to 8.94. The pH was adjusted by adding 1 M NaOH and HCl solutions while recording the values with an electronic pH meter at room temperature. For each sample, media containing a few capsules was transferred into a self-made liquid chamber with a quartz slide as the bottom. All SERS measurements were performed with a Renishaw Raman microscope system. Measurements on capsules were conducted by focusing on the edge of the selected capsule, using a 785 nm laser through a 50× objective lens. For each sample, spectra were collected from ten random points along the edge, with 33 mW laser power, 5 s integration time and two accumulations.

Whole Capsule Imaging

Freshly prepared probe-embedded capsules were transferred into complete cell culture media with different pH. The suspensions were gently shaken for 30 min and subsequently 40 μL of media containing capsules was dropped on a quartz slide surrounded by biocompatible dentist glue, which generated a shallow chamber with a wall 0.5 μm high. The chamber was then covered with a clean quartz square coverslip to confine the movement of the capsule during Raman imaging. A whole capsule was imaged using a 785 nm laser in streamline mode, through a 10× objective lens, with a step size of 20 μm in x-axis and 6.9 μm in y-axis. It should be noted that, the step size in y-axis along the line excitation laser was determined and restricted by the objective lens and CCD sampling. The scanning time for each SERS map was 12-18 min.

pH Determination of Capsules with Live and Dead Encapsulated Cells

Encapsulated cells were grown in low glucose Dulbecco´s modified Eagles´s medium supplemented with inactivated 10% FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic solution, at 37 °C in a 5% CO2/ 95% air atmosphere, and passaged every 2−3 days to

induce cell death. Next, 200 μL of capsule-suspended media from samples with encapsulated cells grown with either high or low glucose content was introduced in a self-made liquid chamber with a quartz slide as bottom. For each capsule, SERS spectra were collected from 20 randomly selected points along the edge, using 33 mW laser power of a 785 nm laser through a 50× objective lens with 5 s integration time. We measured 6 capsules for the dead cell group, 9 capsules for the live cell group.

Data processing

As a routine, all spectral data were preprocessed by cosmic ray removal, baseline subtraction with intelligent fitting mode and smoothing by Savitzky-Golay filter using the Renishaw software Wire 4.4. Thereafter, spectra were processed in batch by a homebuilt program via MATLAB software (R2018a, MathWorks) for calibration curve establishment, pH determination, as well as image reconstruction. The integrated intensity from 1400 to 1450 cm^{-1} (indicating the pH sensitive band, denoted as Iint) was calculated and the ratio of Iint versus the intensity at 1081 cm^{-1} (denoted as I_{1081}) was used for pH determination. The correlation curve between pH and the intensity ratio was fitted to a Boltzmann function and a calibration curve was built for pH measurements. For pH mappings, spectra with intensity at 1081 cm^{-1} below the threshold were filtered out to remove data out of the edge, as those spectra were recorded out of focus, which might influence the accuracy of pH recordings. The remaining spectra were converted into a pH map according to the calibration curve.

Cellular viability Assay of encapsulated cells

Metabolic activity from encapsulated cells, labeled and non-labeled with SERS nanosensor ([Au] = 10, 100 and 1000 μM was quantified by means of a Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich). The number of nanosensors per μm2 were thus 10, 100 and 1000 (~7.8×10^6 to ~7.8×10^8 nanoparticles per capsule, see details in Figure S6). Briefly, 10 μL of microcapsules suspension per well was placed in 100 μL of medium, on 96-well plates. Afterwards, 10 μL of CCK-8 solution was added to each well and incubated for 4 hours at 37 °C. Samples were read on an Infinite M200 (TECAN Trading AG, Switzerland) microplate reader at 450 nm, with a reference wavelength set at 650 nm. At least five wells per sample and three independent experiments were analyzed for each condition.

Statistical analysis

Statistical analysis was performed with IBM SPSS software, version 13.0. Data were expressed as mean ± standard deviation, and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test, when $p < 0.05$. Normality tests were performed to confirm a normal distribution.

z-potential measurements and DLS study before and after polymer coating

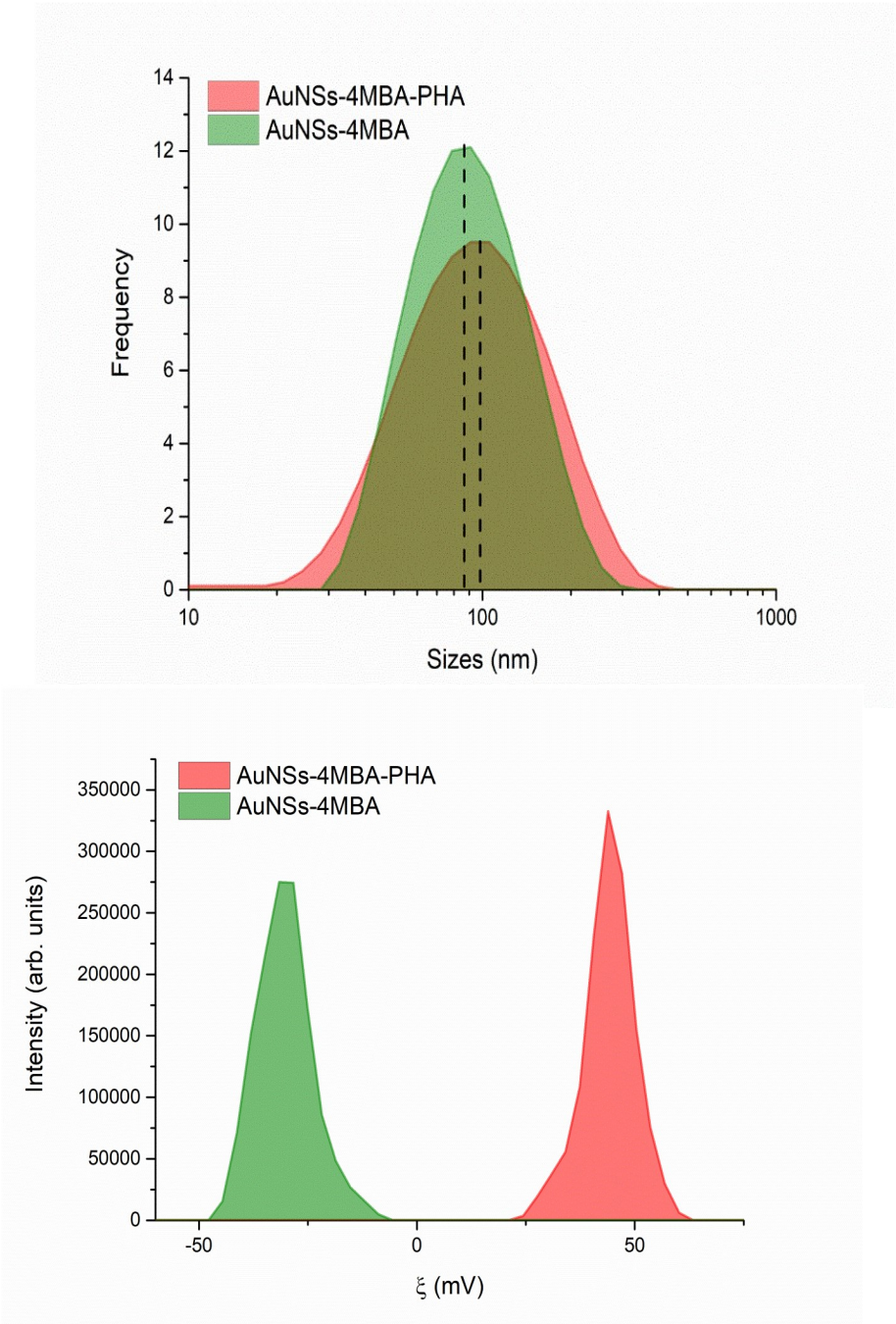

Figure S1. (Upper) DLS size distribution (by intensity) and (Lower) Zeta potential distribution for **pH SERS sensor** before (green) and after PAH coating (pink). Both measurements were performed in Milli-Q water at room temperature.

TEM images of SERS sensor at different magnifications

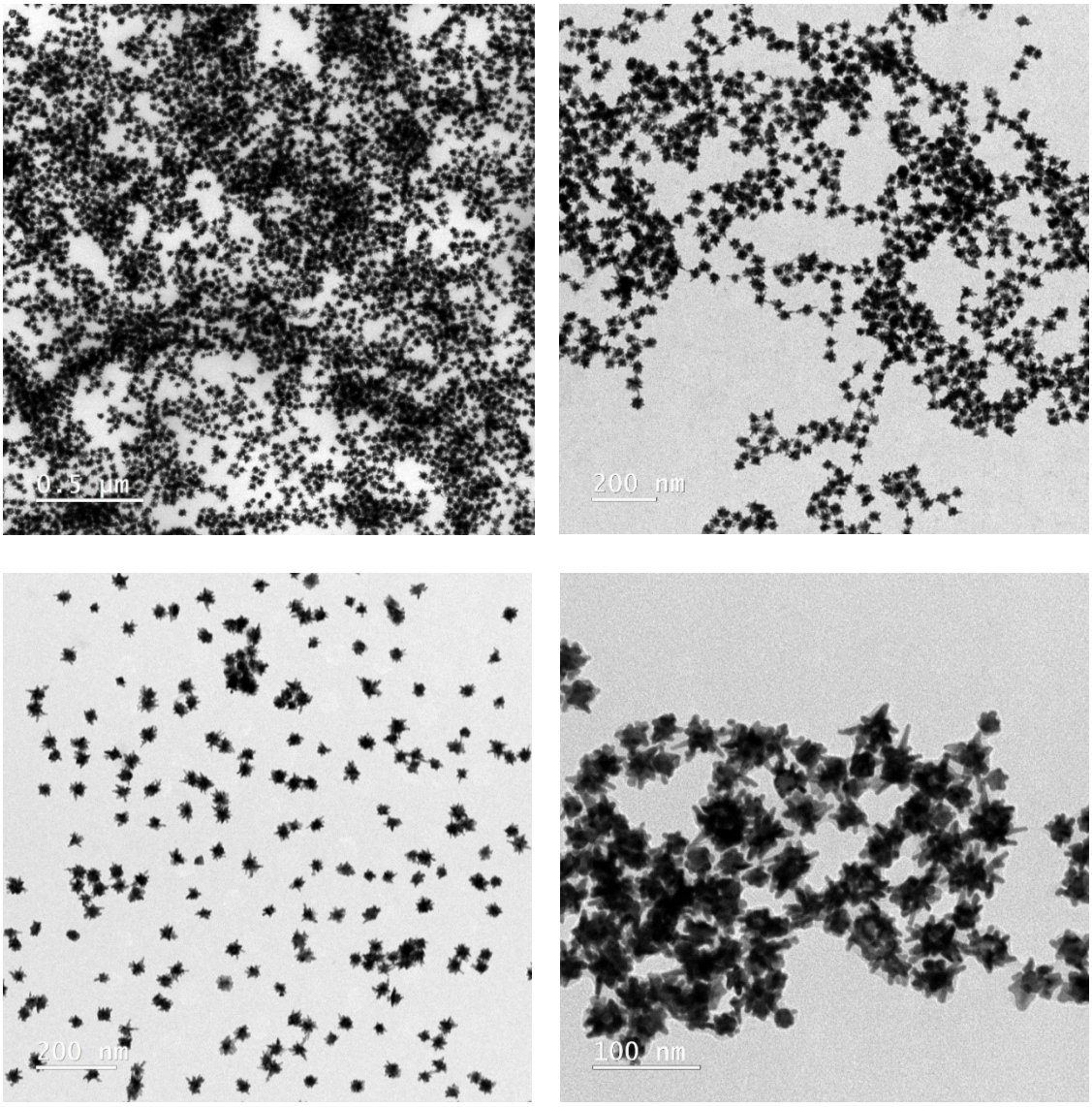

Figure S2. Representative TEM images of pH SERS sensor at different magnifications and concentrations.

STEM-EDX study of labeled microcapsule

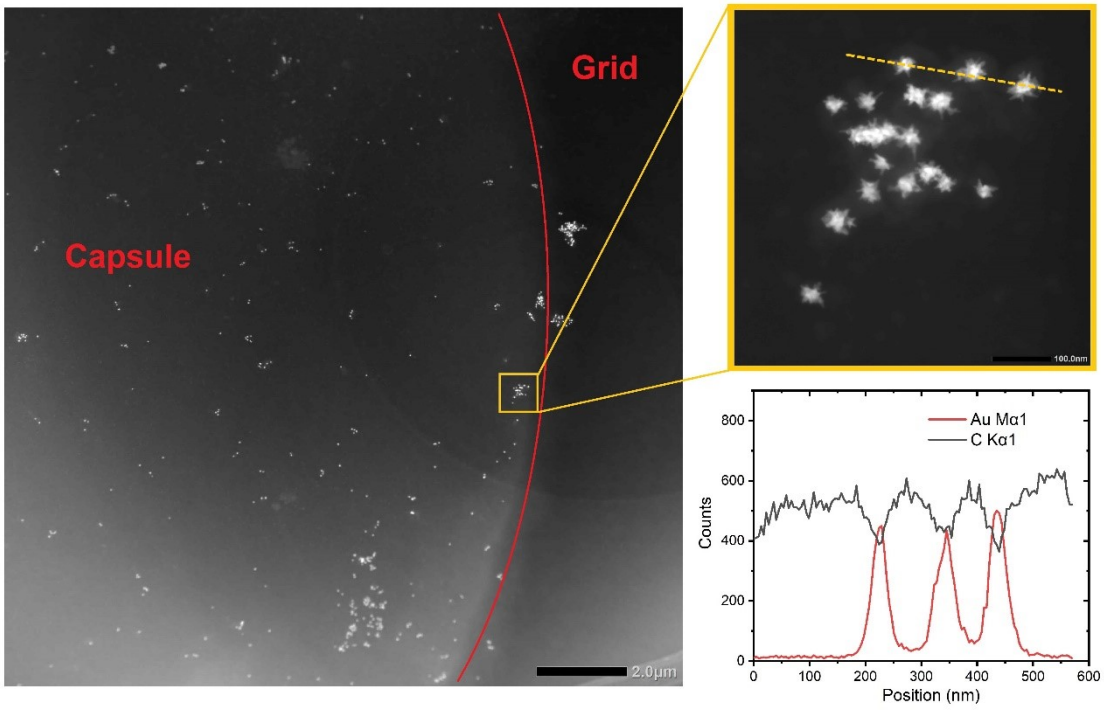

Figure S3. STEM-EDX images of pH SERS sensor labelled microcapsules.

pH calibration curve

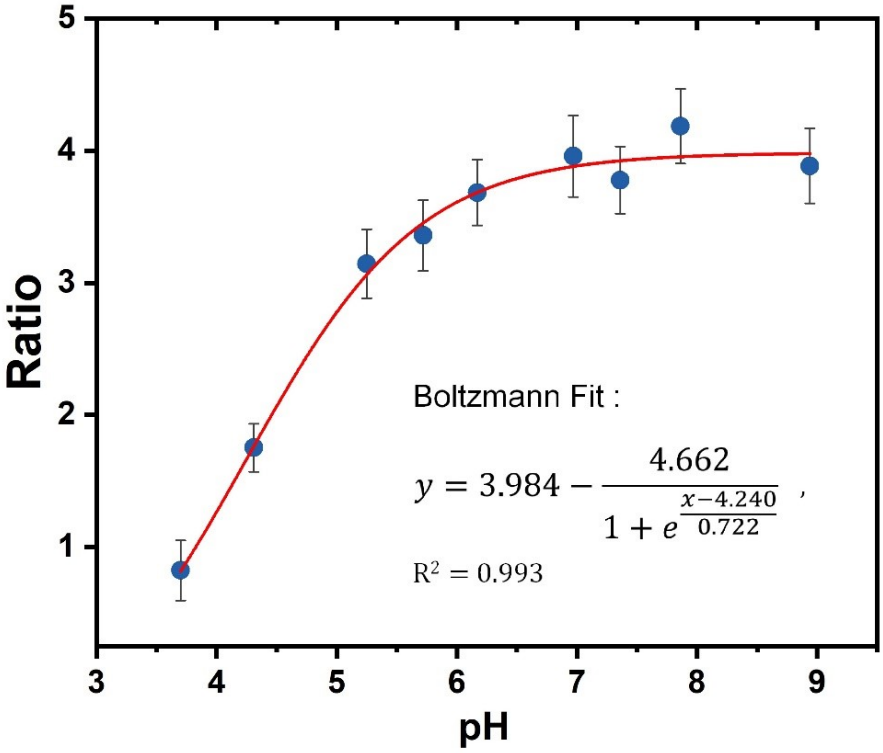

Figure S4. A plot of ratio versus pH and the pH calibration curve fit by Boltzmann equation.

SERS imaging procedure

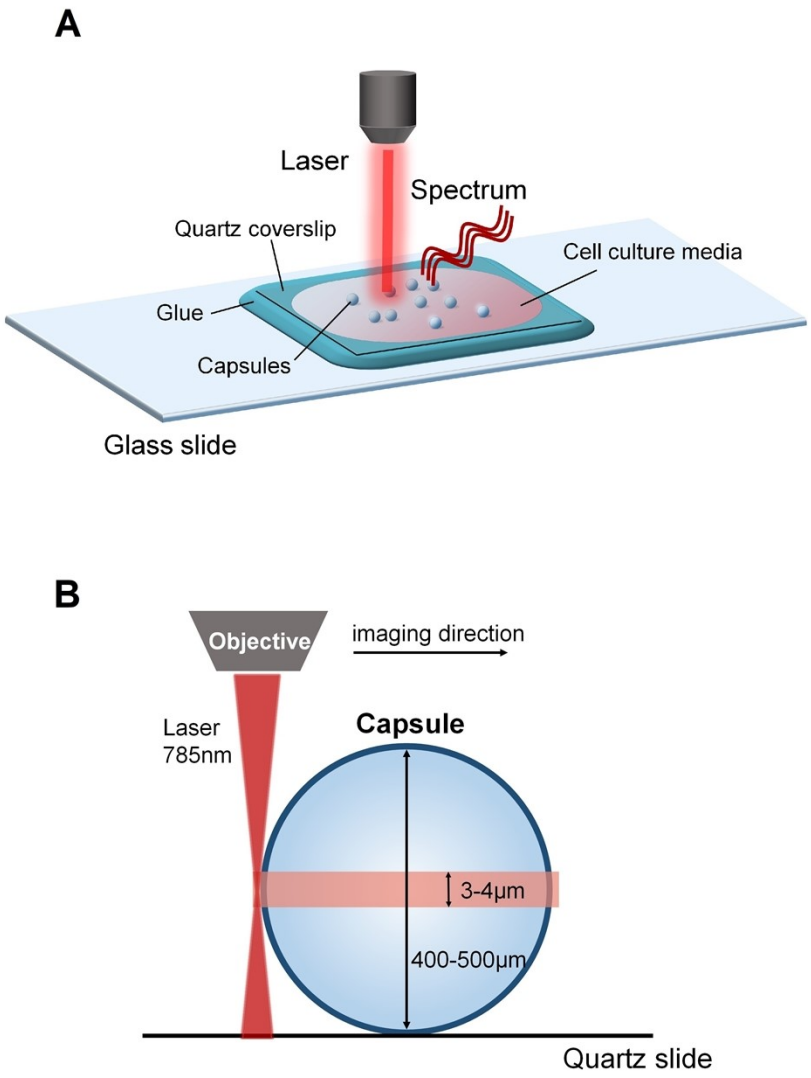

Figure S5. A) The illustration of the home-built setup for capsule measurement and SERS imaging in order to prevent capsule's movement during the observation. B) The lateral view illustration of the imaging on a single capsule.

Cell viability study

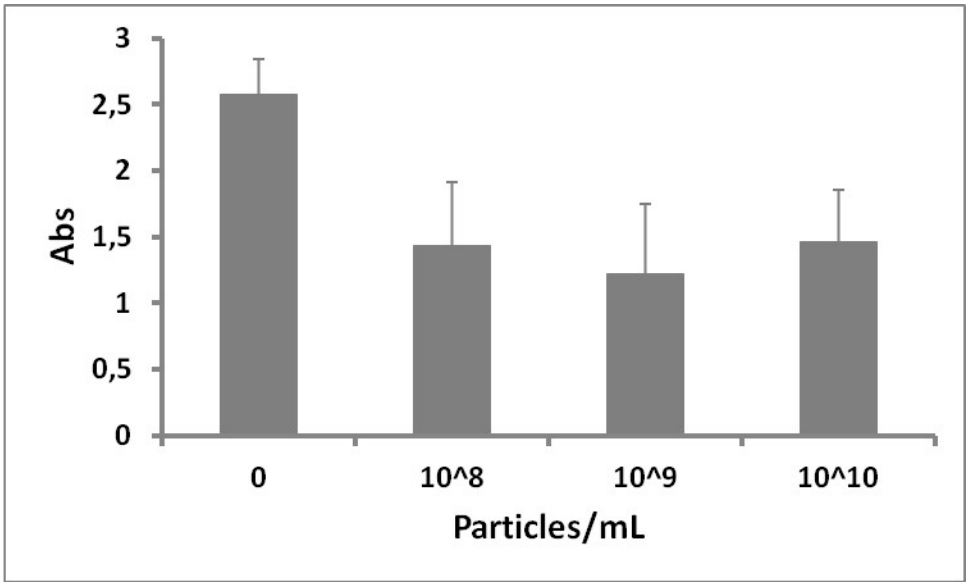

Figure S6. Graphical representation of metabolic activity measured by means of CCK8 assay for microcapsules containing pH nanosensors at different concentrations ([Au] 10-1000 μM, *i.e.* [NPs] 7.8×10^{8} – 7.8×10^{10} nanoparticles/mL) seven days after labelling.

The estimation of the number of AuNSs per capsule is made as follows. Take the labeling density (10 nanoparticles/μm^2) as an example, according to the Au concentration and the size of AuNSs, the concentration of AuNSs was estimated to be 2.6×10^{-12} M for the case of [Au] = 10 μM. Consequently, 500 μL of the solution contains ~7.8×10^{8} AuNSs, namely,

$$N_{AuNS} = 7.8\times10^{8}$$

The diameter of an individual capsule is approximately 500 μm, thus the surface area of the capsule is

$$S_{capsule} = \pi D^2 \approx 3.14\times500^2 = 7.85\times10^{5}\ \mu m^2$$

As an example of SERS nanosensor labeling process, if a 10 μL of capsule suspensions were added to 500 μL of SERS probe solution. Theoretically, according to the principle of close packing, the most capsules per unit volume (μL) of suspensions is 11. We estimated the capsule content in the 10 μL of capsule suspensions were 10, meaning that there were approximately 100 capsules going to be labeled with SERS sensors. Therefore, the number of sensors per capsule would be

$$C_{AuNS/capsule} = 7.8\times10^{8} \div 100 = 7.8\times10^{6}\ /capsule.$$

The sensors labeling density per area on the capsule surface would be

$$D_{AuNS/capsule\ surface} = N_{AuNS} \div (100\times S_{capsule}) = 7.8\times10^{8} \div (100\times7.85\times10^{5}) \approx 10\ /\mu m^2.$$

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2. Enustun, B., Turkevich, J., Coagulation of colloidal Gold, *J. Am. Chem. Soc.* **85**, 3317-3328, (1963).

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