Electronic Supplementary Information

Silica sub-microspheres induce autophagy in an endocytosis dependent manner

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Supplementary Figures

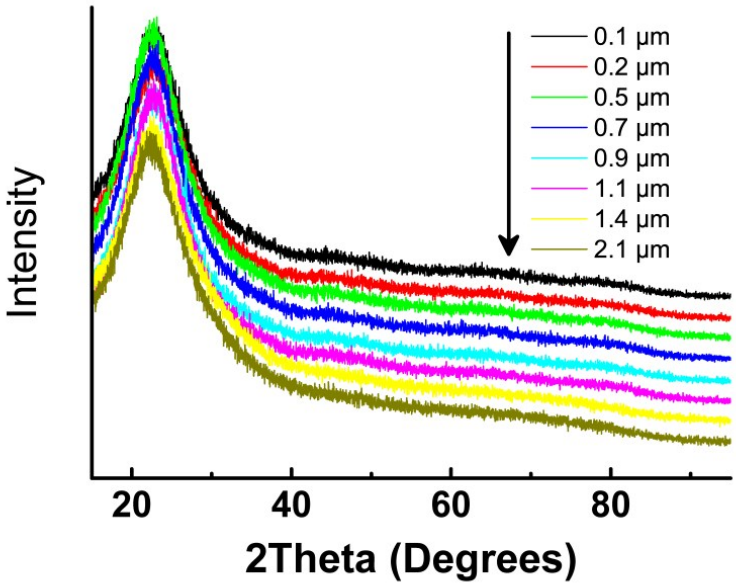

Figure S1. XRD spectra of silica microspheres. The peak at about 22 degree is resulted from the glass background.

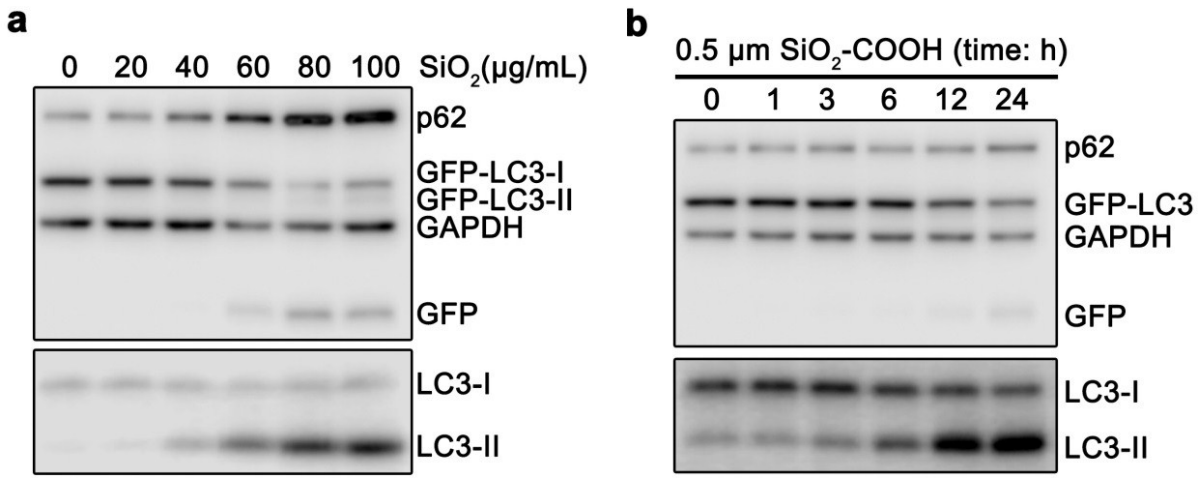

Figure S2. Time and dose effects of silica sub-microspheres inducing autophagy. **a**, Cells were treated with 0~100 μg/mL silica sub-microspheres (0.5 μm in diameter) for 24 h. **b**, Cells were treated with 60 μg/mL silica sub-microspheres (0.5 μm in diameter) for 1~24 h.

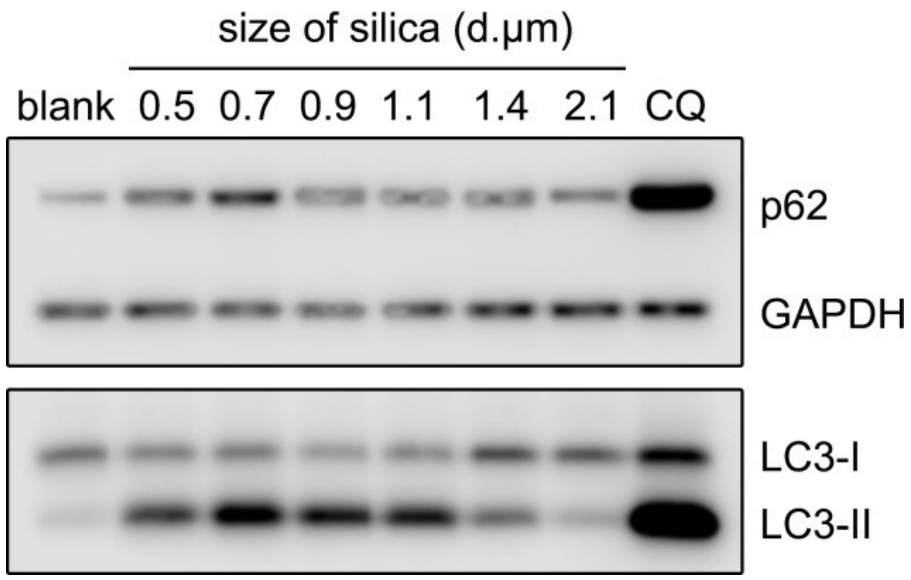

Figure S3. The influence of number concentration on autophagy induction. Cells are treated with silica sub-microspheres at a number concentration of 21 pM for 24 h.

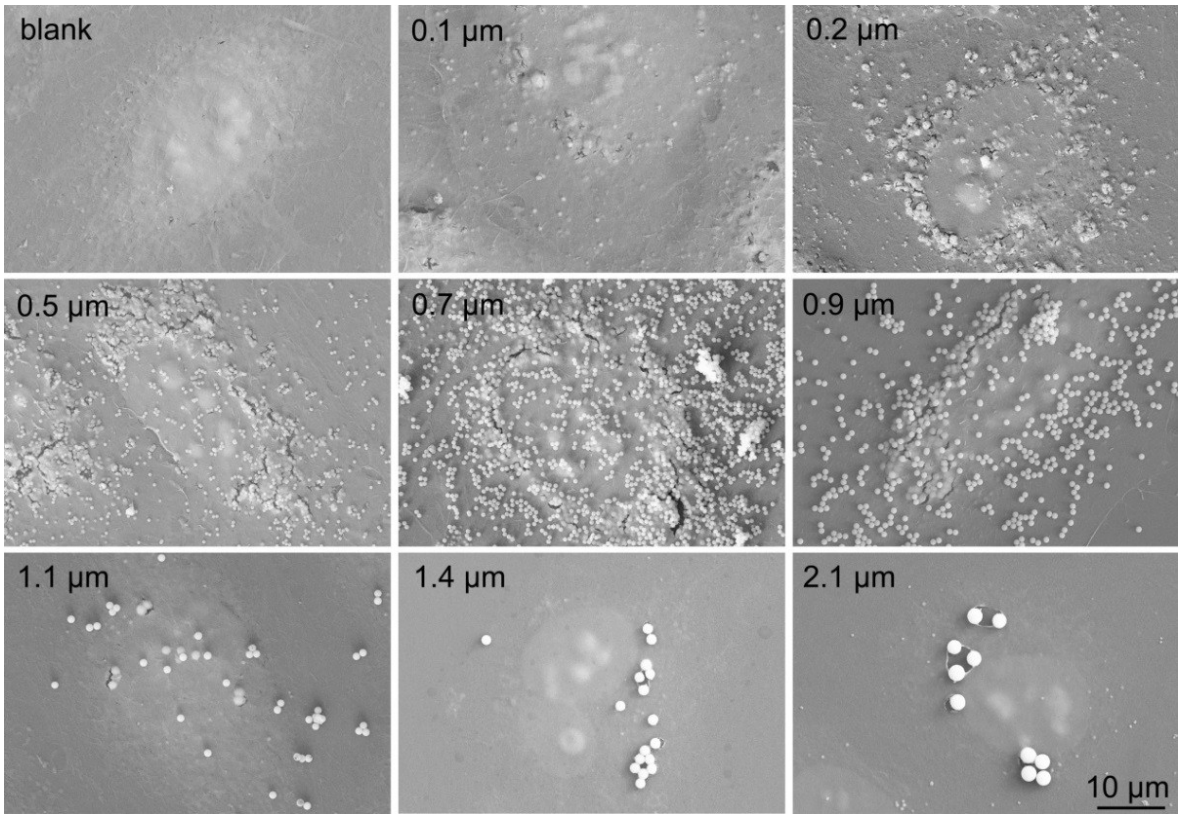

Figure S4. SEM images of cells after treated with various-sized silica sub-microspheres for 24 h. Scale bar is 10 μm.

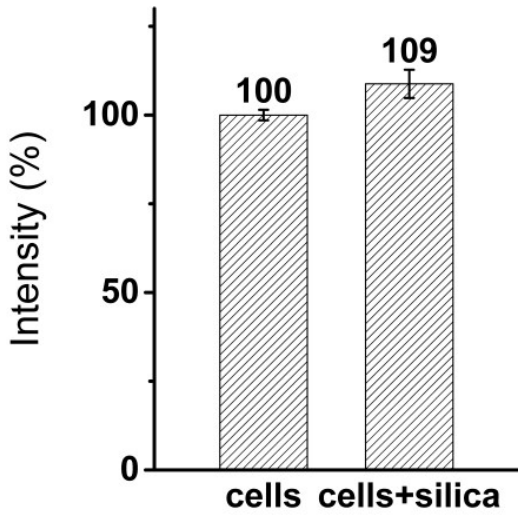

Figure S5. Influence of binding particles on cellular fluorescence. Cell were suspended in complete medium with or without 60 μg/mL fluorescent silica (0.5 μm in diameter) and then analyzed by flow cytometry.

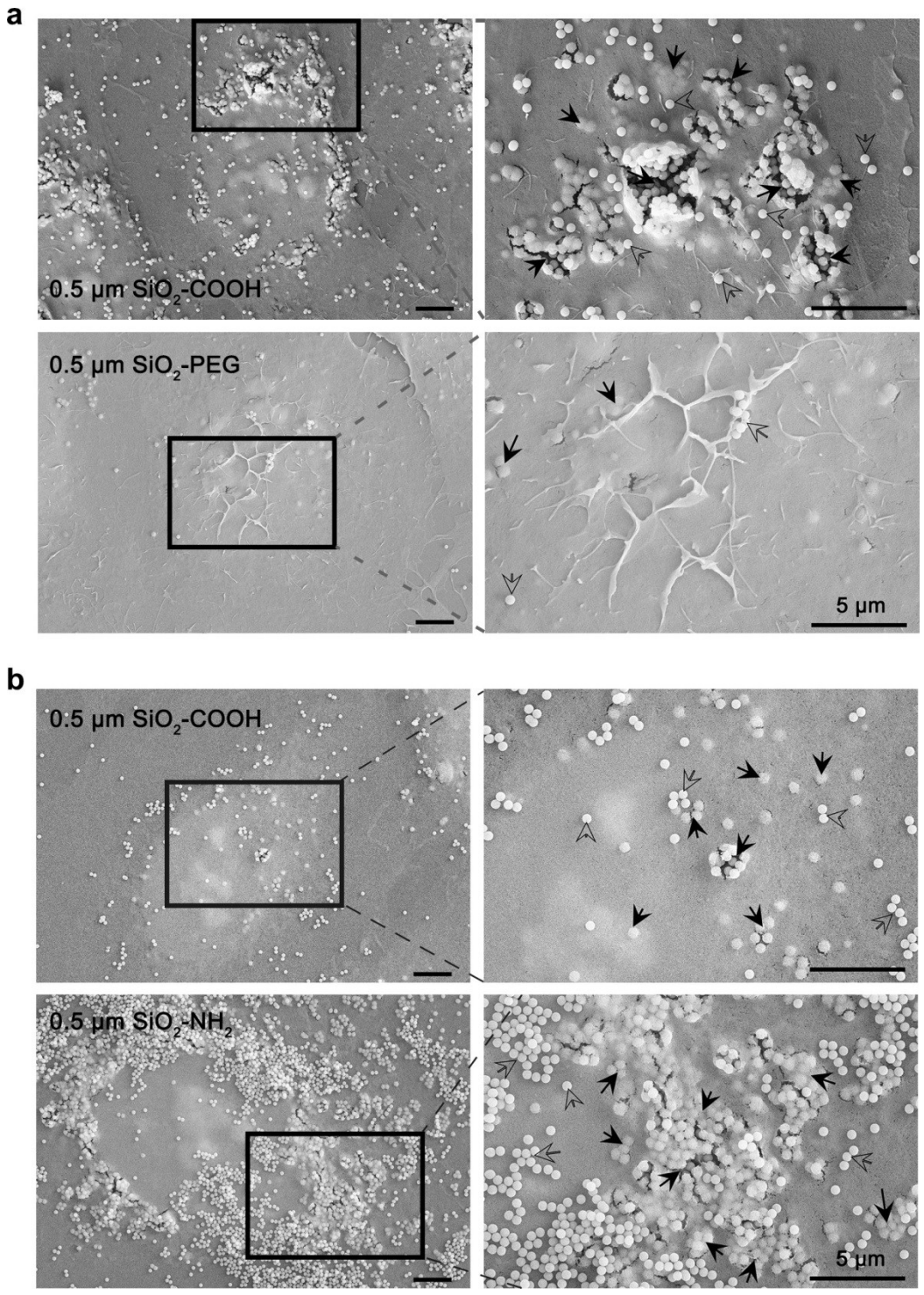

Figure S6. a, Representative SEM images of cells after treated with 0.5 μm SiO_2-COOH or SiO_2-PEG for 24 h. **b**, Cells were treated with 0.5 μm SiO_2-COOH or SiO_2-NH_2 for 6 h. Black solid arrows point to some particles inside cells, which are wrapped by cell membrane and smooth. Blank hollow arrows point to some particles outside cells. All the scale bars are 5 μm.

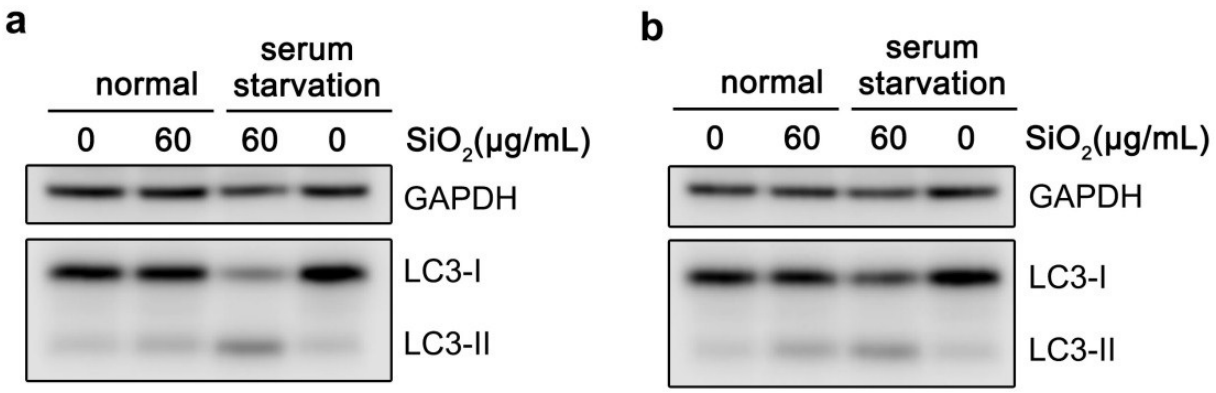

Figure S7. Serum starvation promotes internalization of silica sub-microspheres and subsequent autophagy induction. **a**, Cells were treated with 0.2 μm silica sub-microspheres. **b**, Cells were treated with 1.1 μm silica sub-microspheres.

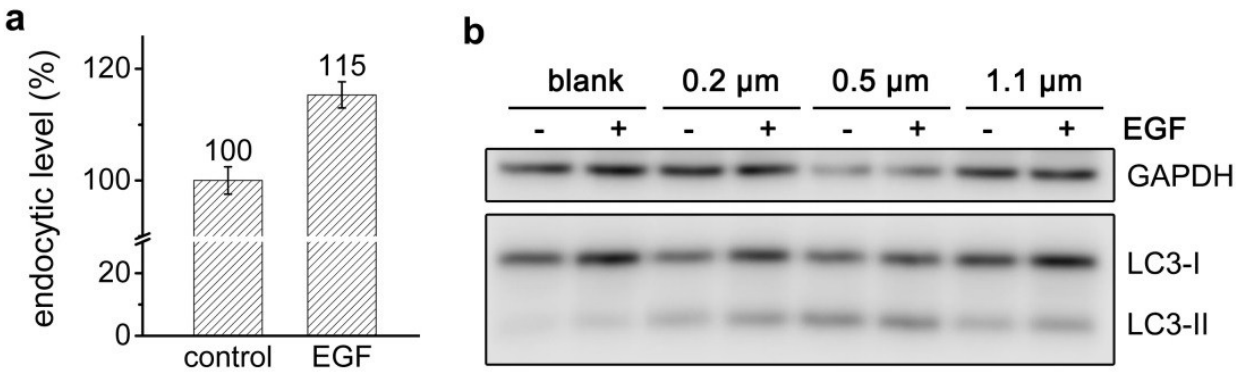

Figure S8. Epidermal growth factor (EGF) facilitates endocytosis of silica sub-microspheres and subsequent autophagy induction. **a**, Flow cytometry analysis of the endocytic levels of 0.5 μm fluorescent silica sub-microspheres in the presence of EGF (10 ng/mL) or not. Data are expressed as mean ± S.D. from three repeated experiments. **b**, Cells were treated with silica sub-microspheres of various sizes in the presence of EGF or not.

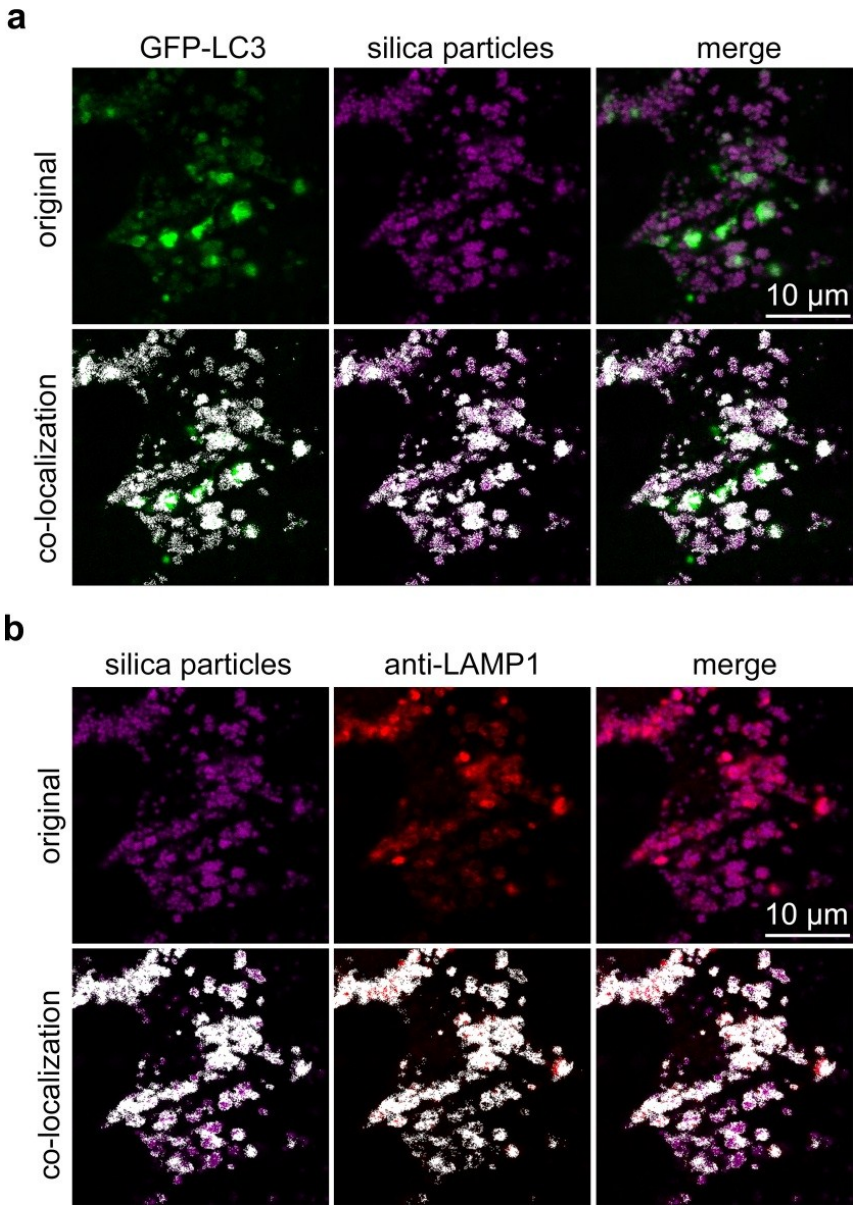

Figure S9. Co-localization of 0.5 μm fluorescent silica sub-microspheres with GFP-LC3 labeled autophagosomes or LAMP1 labeled lysosomes. **a**, 65.3% autophagosomes co-localize with silica and Pearson's coefficient is 0.559. Images in top panel are stained with pseudo color. Images in bottom panel are stained white for co-localized pixels and pseudo color for other pixels. **b**, 67.8% lysosomes co-localize with silica and Pearson's coefficient is 0.642. All the scale bars are 10 μm.

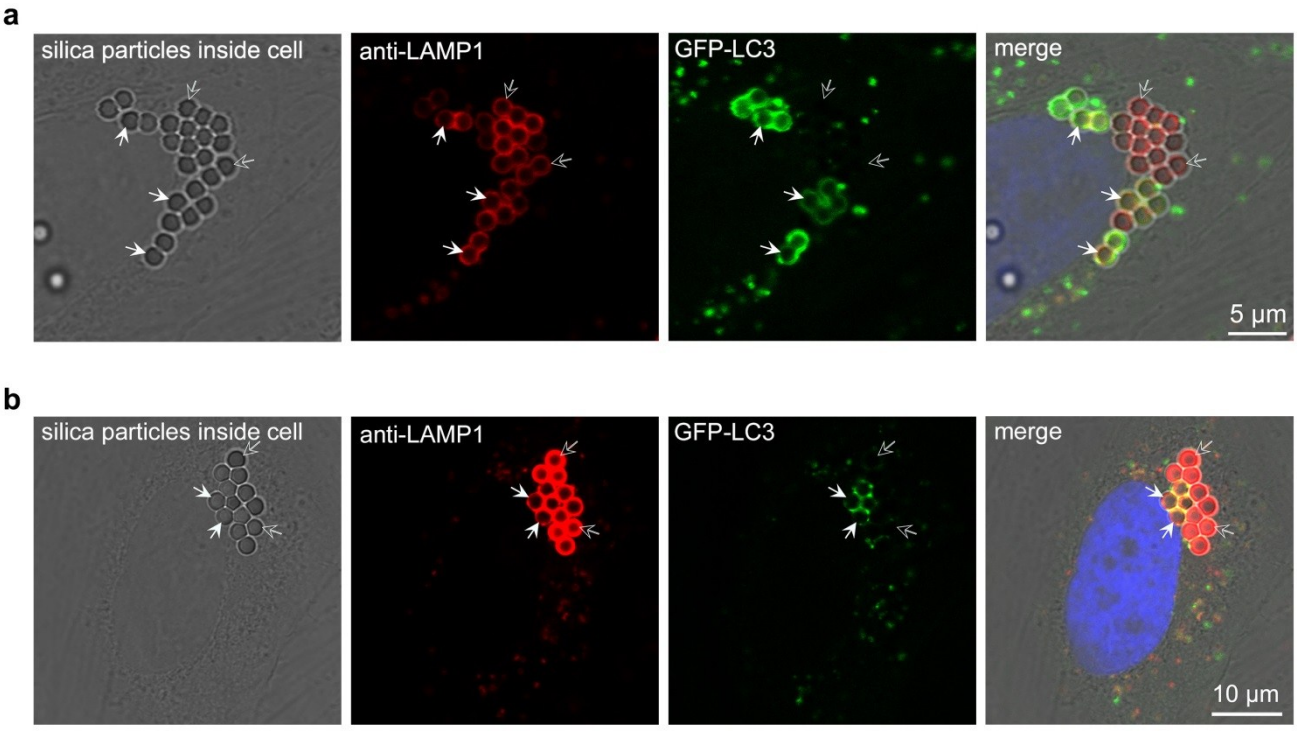

Figure S10. Silica sub-microspheres locate in autophagosomes and/or lysosomes. **a**, 1.4 μm silica sub-microspheres. Scale bar is 10 μm. **b**, 2.1 μm silica sub-microspheres. Scale bar is 5 μm. White solid arrows point to some silica sub-microspheres locate in LAMP-1 (red) and GFP-LC3 (green) dual-labeled autolysosomes. White hollow arrows point to some silica sub-microspheres locate in LAMP-1 single-labeled lysosomes. Nucleus is stained with DAPI (blue).

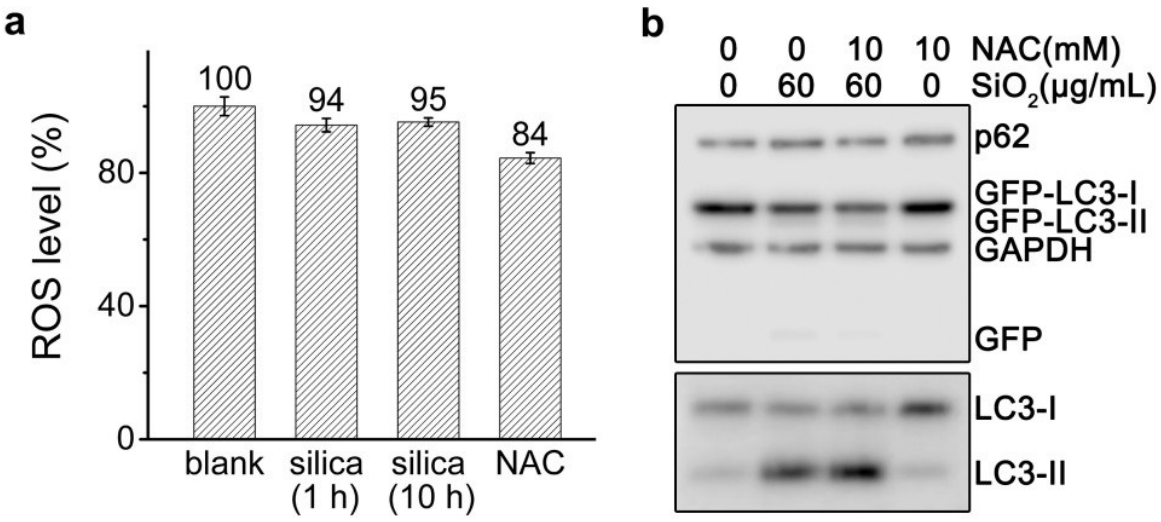

Figure S11. Silica sub-microspheres do not increase ROS generation. **a**, The ROS level was determined by CellROX Green (Invitrogen, Thermo Fisher Scientific) according to the instructions. Cells were treated with 60 μg/mL silica sub-microspheres (0.5 μm in diameter) for 1 h or 10 h, or 5 mM NAC for 0.5 h. Data are expressed as mean ± S.D. from three repeated experiments. **b**, Western blot analysis. Cells were pre-treated with 10 mM NAC for 0.5 h and then incubated with NAC alone or together with silica (0.5 μm in diameter) for another 12 h.

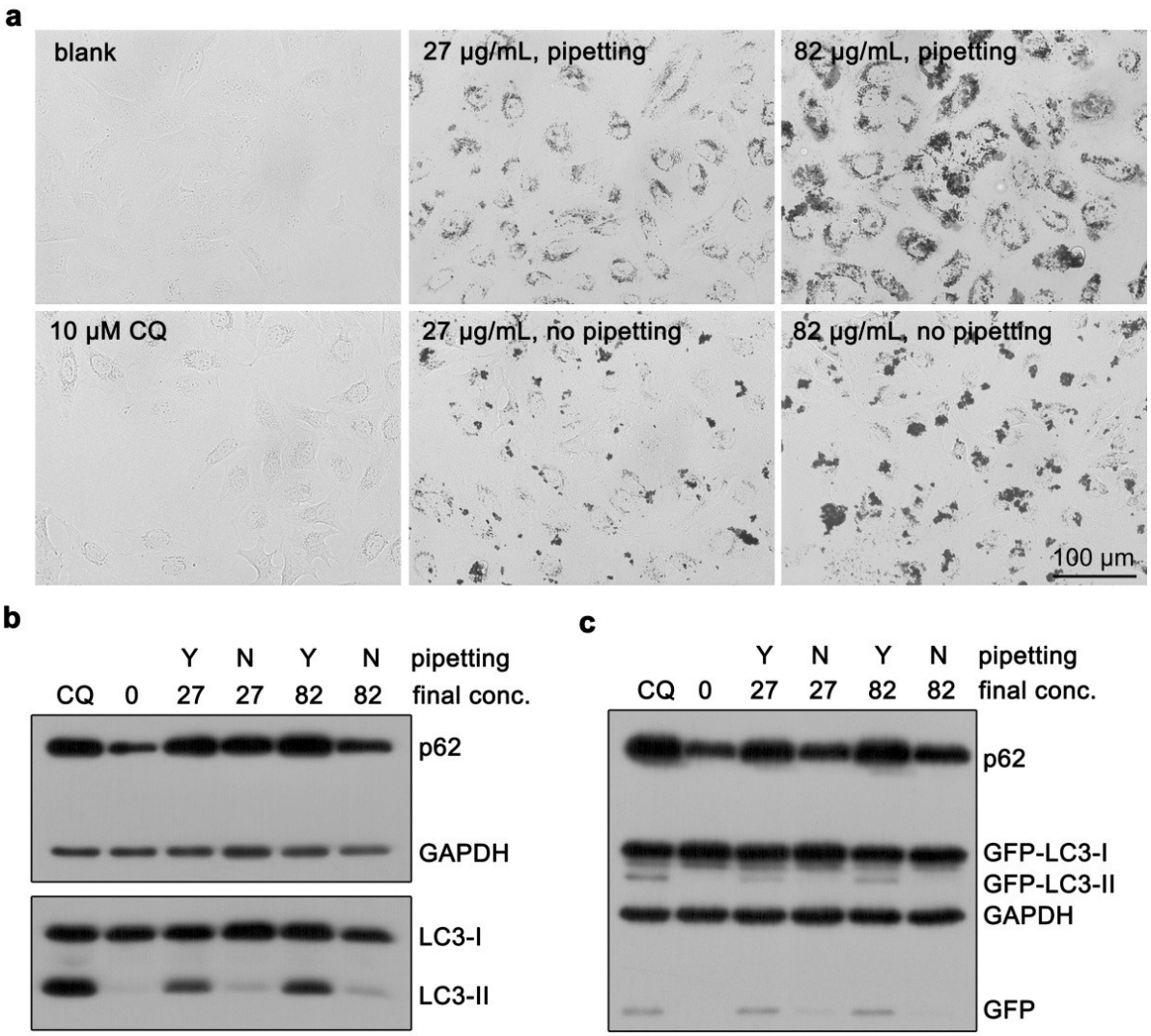

Figure S12. Pipetting[1] reduces the size of aggregation of gold nanoparticles and facilitates autophagy induction. **a**, Bright field images. Cells were treated with cysteine coated gold nanoparticles (12 nm) or chloroquine (CQ) for 24 h. Scale bar is 100 μm. **b**, Western blot analysis of autophagy related proteins.

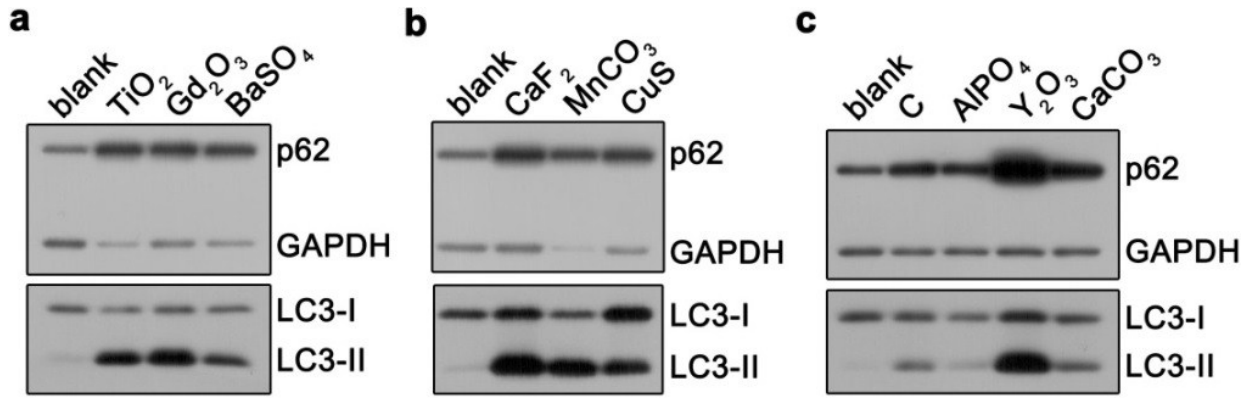

Figure S13. Solid powders or precipitations can induce autophagy. Cells were treated with various sub-micromaterials (1 mg/mL) for 24 h.

Supplementary methods

Synthesis of silica sub-microspheres

Silica sub-microspheres were synthesized using seed-mediated Stöber method.[2,3] In a typical procedure, 134 mg arginine was dissolved in 100 mL ultrapure water with stirring and then 7.5 mL cyclohexane was added. After the solution was heated to 60 °C, 8 mL tetraethoxysilane was added. 24 h later, the solution was diluted to 400 mL with water and heated to 60 °C. After that, 52 g tetraethoxysilane and 58 g cyclohexane were added to the solution. The mixture was stirred at 60 °C and kept for another 48 h. The resulted silica nanoparticles were about 52 nm and used as seeds.

40 mL seed solution and 40 mL 25% ammonium hydroxide were added to 400 mL water. The solution was stirred at room temperature. Then 40 mL tetraethoxysilane was added at a rate of 50 μL/min. The solution was stirred for another 6 h. Then 0.1 μm silica sub-microspheres were obtained and they could be used as seed for growing 0.2 μm silica sub-microspheres. This process was repeated to obtain 0.2, 0.5, 0.7, 0.9 and 1.1 μm silica sub-microspheres. For growth of 0.5 μm or larger particles, ammonium hydroxide was replaced by arginine, which could reduce secondary nucleation. In a typical procedure, 80 mL solution containing 20 mg arginine and 20 mL solution containing 0.2 μm silica sub-microspheres was added to 360 mL ethanol. The mixed solution was heated to 45 °C and 28 g tetraethoxysilane was added. The mixture was stirred at 45 °C for 12~24 h. For growth of 0.7~1.1 μm particles, tetraethoxysilane should be added in portions every 1~2 h. 1.4 μm and 2.1 μm silica sub-microspheres were purchased from Lebeisi Company due to the tedious procedures. All the samples could be purified by low speed centrifugation if necessary.

Fluorescent labeled silica sub-microspheres were synthesized by the same procedure except adding <0.1% silanized rhodamine. In a typical procedure, 5 mg rhodamine B isothiocyanate reacted

with 5 μL 3-Aminopropyltriethoxysilane in 1 mL ethanol with gentle shake for 6~12 h. The mixture was added together with TEOS (less than 1:1,000 in molar ratio) in the regrowing procedure.

Surface modification of silica sub-microspheres

Modification with carboxylate was performed under basic conditions. In a typical procedure, 2 g 0.5 μm silica sub-microspheres were dispersed in 60 mL water. 100 μL 25% carboxyethylsilanetriol sodium salt and 400 μL 25% ammonium hydroxide were added. The solution was stirred and heated to 70 °C. After 12~24 h, silica sub-microspheres were collected by centrifugation and washed with ultrapure water for four times.

Modification with PEG was performed as the same procedure while the amount of mPEG1000 tetraethoxysilane should be increased by fourfold.

Modification with amino groups was performed under acidic conditions. In a typical procedure, 1 g 0.5 μm silica sub-microspheres were dispersed in 60 mL ultrapure water. Then 0.3 mL acetic acid and 0.2 mL 3-[2-(2-Aminoethylamino)ethylamino]propyl- trimethoxysilane were added. The solution was stirred at room temperature for 10 min and then heated to 70 °C. After 12~24 h, silica sub-microspheres were collected by centrifugation and washed with ultrapure water for four times.

Before incubated with cells, all the silica sub-microspheres were sterilized in 75% ethanol at 70 °C for 1 h and then washed four times with sterile water.

Determine the concentration of silica sub-microspheres

We determined the concentration of silica sub-microspheres by molybdenum blue spectrophotometry. For the detail procedure please refer to our previous publication.[1] All concentrations of silica sub-microspheres were expressed as the concentration of SiO_2.

Western blot

The antibodies are listed as follows: Anti-LC3B (1/3,000, Abcam, ab51520), Anti-GAPDH (1/10,000, Abcam, ab128915), Anti-SQSTM1/p62 (1/20,000, Abcam, ab109012), Anti-GFP (1/5,000, Abcam, ab137827), Anti-LAMP1 (1/1,000, Cell Signaling Technology, #9091, D2D11 XP). Anti-mTOR (1/1,000, Abcam, ab134903), Anti-p-mTOR(Ser2448) (1/1,000, Cell Signaling Technology, 5536S), Anti-β-tubulin (1/5,000, Abcam, ab68193), Anti-p-p70 S6K(Thr389) (1/1,000, Cell Signaling Technology, 9234S), Anti-p70S6K (1/2,000, Cell Signaling Technology, 2708P), Anti-FOXO3A (1/4,000, Abcam, ab53287), Anti-p-FOXO3A(Ser253) (1/1,000, Abcam, ab154786), Anti-p-AKT(Ser473) (1/1,000, Cell Signaling Technology, 4060P), Anti-AKT (1/1,000, Cell Signaling Technology, 4691P), Goat polyclonal Secondary Antibody to Rabbit IgG – H&L (horse radish peroxidase, HRP) (1/5,000, Abcam, ab97051).

Electron microscopy imaging of cells

For SEM and TEM imaging of cells, please refer to our previous publication[1].

Colocalization analysis of confocal images

Confocal images were exported as tif format. Colocalization analysis was performed using ImageJ plugin JACoP[4] (http://rsb.info.nih.gov/ij/plugins/track/jacop.html). Threshold values were set automatically to calculate Manders' co-localization coefficients (M1 and M2) and Pearson's correlation coefficient.

Other cell experiments

The procedure of synthesis and modification of cysteine coated gold nanoparticles were similar to that of Au@DMSA[1].

When using serum starvation to promote endocytosis, cells were cultured in MEM medium with

60 μg/mL silica sub-microspheres while without fetal bovine serum. After 4 h, replaced it with MEM medium with fetal bovine serum while without silica sub-microspheres and continued to incubate for 20 h.

When using inhibitors of endocytosis, cells were pre-treated with inhibitors for 4 h and then together with silica sub-microspheres for another 4 h. While cytochalasin D should be removed after 0.5 h pre-treatment.

Supplementary References

1 Huang, D., Zhou, H. & Gao, J. Nanoparticles modulate autophagic effect in a dispersity-dependent manner. *Sci. Rep.* **5**, 14361 (2015).

2 Watanabe, R. *et al.* Extension of size of monodisperse silica nanospheres and their well-ordered assembly. *J. Colloid Interf. Sci.* **360**, 1-7 (2011).

3 Hartlen, K. D., Athanasopoulos, A. P. T. & Kitaev, V. Facile Preparation of Highly Monodisperse Small Silica Spheres (15 to >200 nm) Suitable for Colloidal Templating and Formation of Ordered Arrays. *Langmuir* **24**, 1714-1720 (2008).

4 Bolte, S. & CordeliÈRes, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microscopy* **224**, 213-232 (2006).