Supporting information for

Paradox of nobility: How gold TEM grid disrupts cellular integrity while nickel preserves stability

Yongdeok Ahn,[+,a] Juhyeong Cho,[+,a] Siwoo Jin,[a] and Daeha Seo*[,a]

[a]Department of Physics and Chemistry, DGIST, Daegu, 42988, Republic of Korea

[+]These authors contributed equally to this work.

Correspondence should be addressed to D.S. (livewire@dgist.ac.kr)

Experimental sections

Chemicals and Materials

For general experiments. Acetic acid (99.7%; Sigma-Aldrich), acetone (99%; Alfa Aesar), alconox detergent (Sigma-Aldrich), ethanol (EtOH, 96%; Alfa Aesar), sodium hydroxide beads (NaOH, ≥97 %; Sigma-Aldrich), deionized water (DI water, 18.2 MΩ), Amplex red (Thermo Fisher Scientific) Cu EM grid (Formvar/Carbon 200 mesh; TED Pella), Au EM grid (Formvar/Carbon 200 mesh; TED Pella), Ni EM grid (Formvar/Carbon 200 mesh; TED Pella).

For cell experiments. Cell lines (U2OS, SK-N-SH; Korean Cell Line Bank), Collagen I (3.6 mg/mL, rat tail; Santa Cruz Biotechnology), DCFDA/H2DCFDA cellular ROS assay kit (Abcam), Dulbecco's modified Eagle's medium (DMEM, high glucose; Biowest), fetal bovine serum (FBS; Biowest), ethanol (pure, 200 proof, anhydrous, ≥99.5%; Sigma-Aldrich), ER-Tracker™ Green (glibenclamide BODIPY® FL; Thermo Fisher Scientific), glass-bottom dishes (MatTek, 10 mm well, 35 mm dish), Hank's Balanced Salt Solution with calcium and magnesium (HBSS/Ca/Mg; Gibco). LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific), osmium tetroxide (99.8%, Sigma-Aldrich), penicillin-streptomycin solution 100X (Biowest), Dulbecco's phosphate-buffered saline (D-PBS; Biowest), 0.25% trypsin-EDTA (Biowest), sodium cacodylate (BioXtra, ≥98%; Sigma-Aldrich).

Methods

Optical microscopy. BF and fluorescence microscopy were conducted using an inverted microscope (Nikon, ECLIPSE Ti2-E) equipped with a perfect focus system (PFS, TI2-N-ND-P), a motorized stage (TI2-S-SE-E), and an electron-multiplying charge-coupled device (EM CCD, Andor, iXon Ultra 888). A stage-top-incubator controlling temperature, humidity, and CO_2 concentration (Okolab, UNO-T-H-PREMIXED) was utilized for live cell imaging experiments. An LED lamp (Nikon, TI2-D-LHLED) and mercury lamp (Nikon, C-HGFIE Intensilight) served as light sources for BF and fluorescence imaging, respectively. BF and fluorescence images were acquired using 10x, 20x (Nikon, CFI Plan Apochromat, 10x: 0.45 NA, 20x 0.75 NA), and 100x objective lenses (Nikon, oil immersion, CFI Apochromat TIRF, 1.49 NA).

Use of Collagen I solution. Collagen I solution was prepared by diluting collagen I (3.6 mg/mL) to a concentration of 50 µg/mL utilizing 0.02 N acetic acid. Following agitation for 2 h, the solution was filtered through a PTFE filter with a 0.2 µm pore size. Prior to cell plating, 100 µL of the collagen solution was incubated for 30 minutes, subsequently followed by three washes with buffer or medium.

Cell lines. Cells (U2OS, bEND5, bEND3, SK-N-SH) were cultured in a T25 flask under conditions of 37 °C and 5% CO_2 in a humidified incubator, utilizing DMEM supplemented with 10% FBS, 100 unit/mL penicillin, and 100 µg/mL streptomycin. The cells were subcultured every 3-4 days. For observation via optical microscopy, appropriate quantities of cells were plated on collagen I-coated glass or TEM grid (Cu, Au, Ni) to achieve 70% confluence. Cell images were subsequently acquired using optical microscopy.

LIVE/DEAD assay to measure cell viability. To assess cell viability on collagen I-coated glass or TEM grids after 1 day, the plated cells were subjected to treatment with a mixture of 2 mL of LIVE/DEAD working solution

containing 1 µM calcein acetoxymethyl ester (Calcein-AM) and 2 µM ethidium homodimer-1 (EthD-1) in D-PBS. Following a 45-minute incubation period, fluorescence images of the cells were acquired using the green and red channels corresponding to live and dead cells, respectively.

DCFDA assay to measure ROS activity within the cell. To visualize the reactive oxygen species (ROS) generation in cells, the plated cells were incubated for 24 hours. The cells were subsequently treated with 100 µL of 25 µM 2',7'-dichlorofluorescin diacetate (DCFDA) solution for 45 min at 37 °C under light-protected conditions. Following this, the cells were rinsed with phosphate-buffered saline (PBS) and exposed to 2 mL of phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS), penicillin, and streptomycin. The culture dish was then placed on a pre-warmed microscope stage. The cells were imaged using the green fluorescence channel to quantify the fluorescence signal for the DCFDA assay.

ICP-MS measurement. To quantitatively analyze the generation of Cu^{n+} (n=1 or 2) induced by cell culture media (DMEM) incubation, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (NexION 2000, Perkin Elmer) measurements were conducted. After incubation, the cell culture media was collected, and subsequently treated with a mixture of HCl and HNO_3 at a volumetric ratio of 3:1. The resulting mixture was heated at 80 ˚C for 2 h, followed by ultrasonication for an additional 3 h.

Amplex red hydrogen peroxide/peroxidase assay. To quantify the generation of reactive oxygen species (ROS), specifically hydrogen peroxide, from collagen-coated electron microscopy (EM) grids, the grids were incubated in 100 µL of 1 µM Amplex red in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) for 24 h at 37 °C in the absence of light. Subsequently, the fluorescent signal of resorufin was immediately measured using a multimode microplate reader (Agilent, Synergy H1) with excitation at approximately 550 nm and emission detection at approximately 590 nm.

Labelling of the ER. To visualize the cellular endoplasmic reticulum using fluorescence microscopy, the plated cells were stained with an ER-Tracker Green. The cells were rinsed with Hank's Balanced Salt Solution with calcium and magnesium (HBSS), then incubated in a prewarmed 5 µM staining solution diluted in HBSS for 1 h at 37 °C in the absence of light. Following replacement with fresh HBSS, the fluorescent signal was immediately observed using optical microscopy.

XPS and FT-IR measurement of the EM grids. The oxidative state of metal and carbon film was investigated utilizing X-ray photoelectron spectroscopy (XPS) (ESCALAB 250Xi, Thermo Scientific) and Fourier-transform infrared spectroscopy (FT-IR) (Nicolet Continuum, Thermo Scientific) analysis. Following the measurement of the oxidative states of electron microscopy (EM) grids without any treatment, the same EM grids were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 µg/mL streptomycin for 24 h at 37 °C under flowing 5% of CO2. The incubated EM grids were subsequently washed with deionized (DI) water twice and dried completely to remove the remaining salts, then re-measured using XPS and FT-IR under identical conditions. The obtained XPS data were analyzed using XPS peak 4.1 software.

AFM measurement. The thickness of Collagen I on EM grid was measured utilizing atomic force microscopy (AFM) (XE-150, Park Systems) and a non-contact cantilever. The AFM images obtained of Collagen I-coated Ni EM grid were analyzed using XEI software (Park Systems).

TEM measurement. Fixation of cells was conducted for TEM observation. Cells loaded on the EM grid were washed with 0.2 M of sodium cacodylate buffer (pH 7.2) within 30 s and primarily fixed using a mixture of 2% formaldehyde and 2.5% glutaraldehyde in sodium cacodylate buffer for 2 h at room temperature. The primarily fixed cells were washed with the buffer more than 3 times, and secondary fixation was conducted using 1% osmium tetroxide for 40 min at room temperature and washed with the buffer more than 3 times. The fixed cells were dehydrated using 30, 50, 70, 80, 90, 95, and 100% of ethanol (pure). The 70% step was conducted at 4 °C for 30 min. The 95% step was performed twice, each for 5 min, and the 100% step was performed 3 times, each for 3 min at room temperature. Except for the 70, 95, and 100% steps, all steps were performed for 7 min at room temperature. The cells were stained using 7% uranyl acetate/ethanol solution for 10 min and washed with ethanol 3 times within 3 min. HMDS diluted with pure ethanol to 10wt% was applied to the stained cells for 30 s. After fixation, cells loaded on the EM grid were examined by bio-TEM (FEI, Tecnai G2 F20 TWIN TMP) at an acceleration voltage of 80kV.

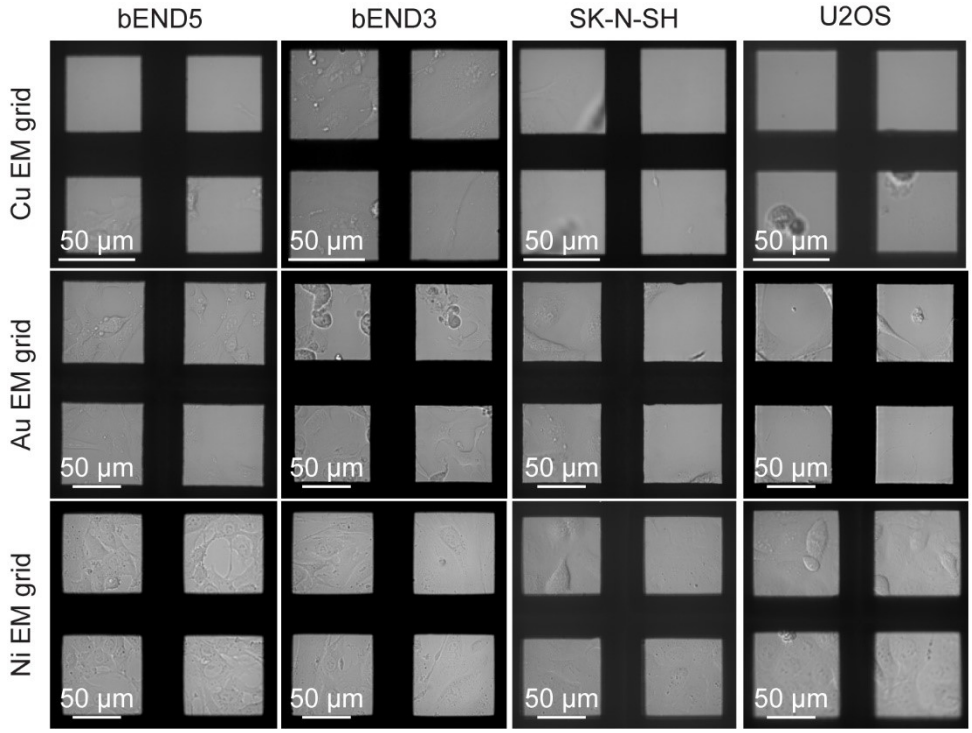

Fig. S1. BF microscopy images of cells cultured on various EM grids. The images depict bEND5, U2OS, bEND3, and SK-N-SH cells directly cultured on Cu, Au, and Ni EM grids. Nickel EM grids provided the most stable environment for cell growth.

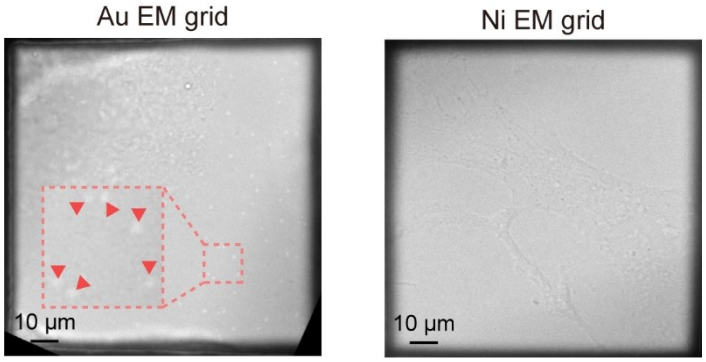

Fig. S2. BF microscopy images of U2OS cells on Au and Ni EM grids. Red arrows indicate debris accumulation, suggesting cellular stress. In contrast, the Ni EM grid exhibits a clean surface with well-preserved cells, indicating a more stable environment.

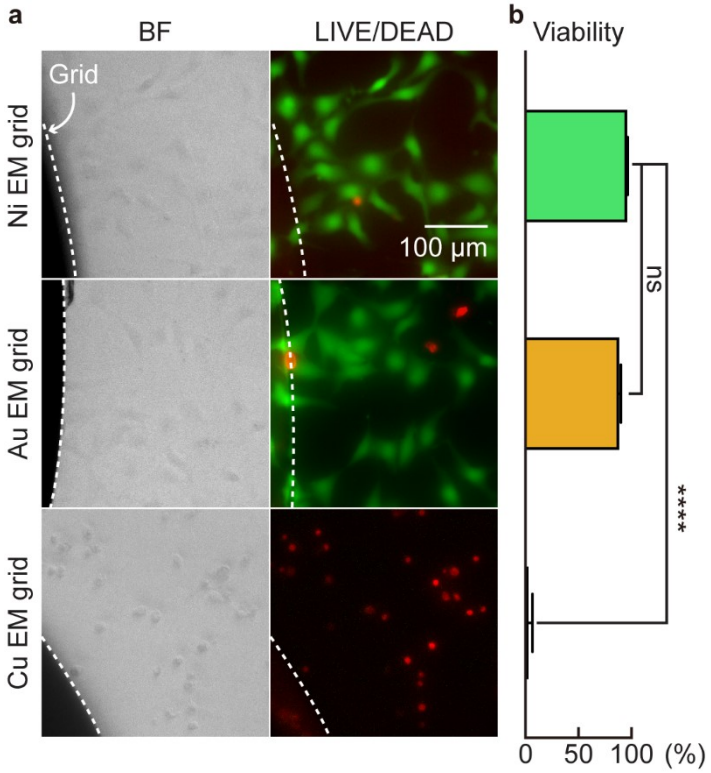

Fig. S3. Viability assessment of cells outside EM grids. (a) BF and Live/Dead assay on fluorescence microscopy image of U2OS cultured on different EM grids. Viable cells appear green, while non-viable cells appear red. The white dashed line demarcates the boundary between the EM grid and the surrounding area. (b) Quantification of cell viability for each EM grid. Ni and Au EM grids show no significant difference, while Cu EM grids exhibit significantly lower viability. ns: not significant; ****p<0.0001.

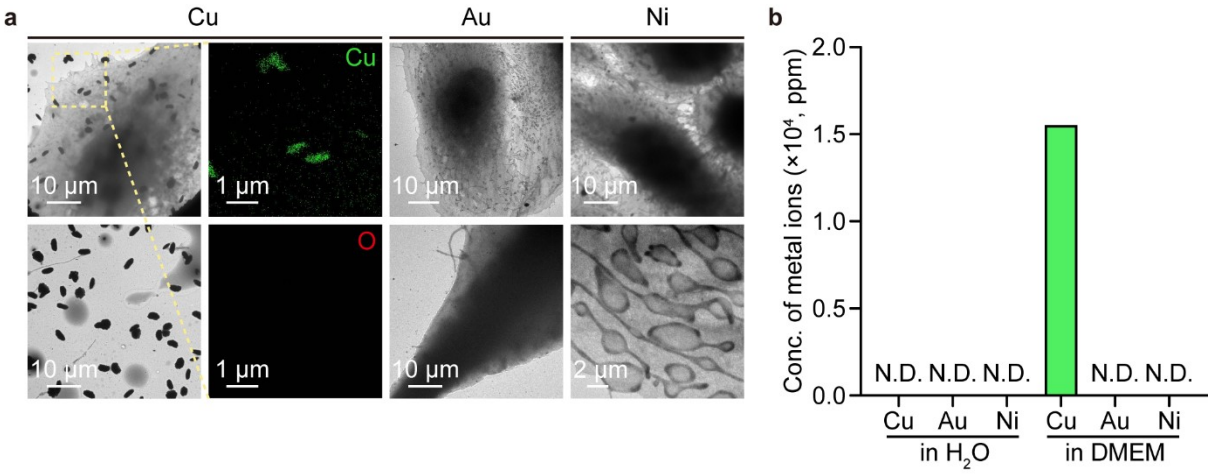

Fig. S4. Metal ion elution from Cu EM grids. (a) Electron microscopy images of U2OS on each EM grid after cell culture. The yellow dashed box indicates Cu crystals, subsequently confirmed by EDS mapping (Cu in green, O in red). In contrast, Au and Ni EM grids demonstrate no significant structural degradation. (b) Quantification of metal ion elution in culture media utilizing ICP-MS analysis. Cu EM grids release a substantial amount of Cu ions, whereas Au and Ni EM grids exhibit not detectable (N.D.) metal ion elution.

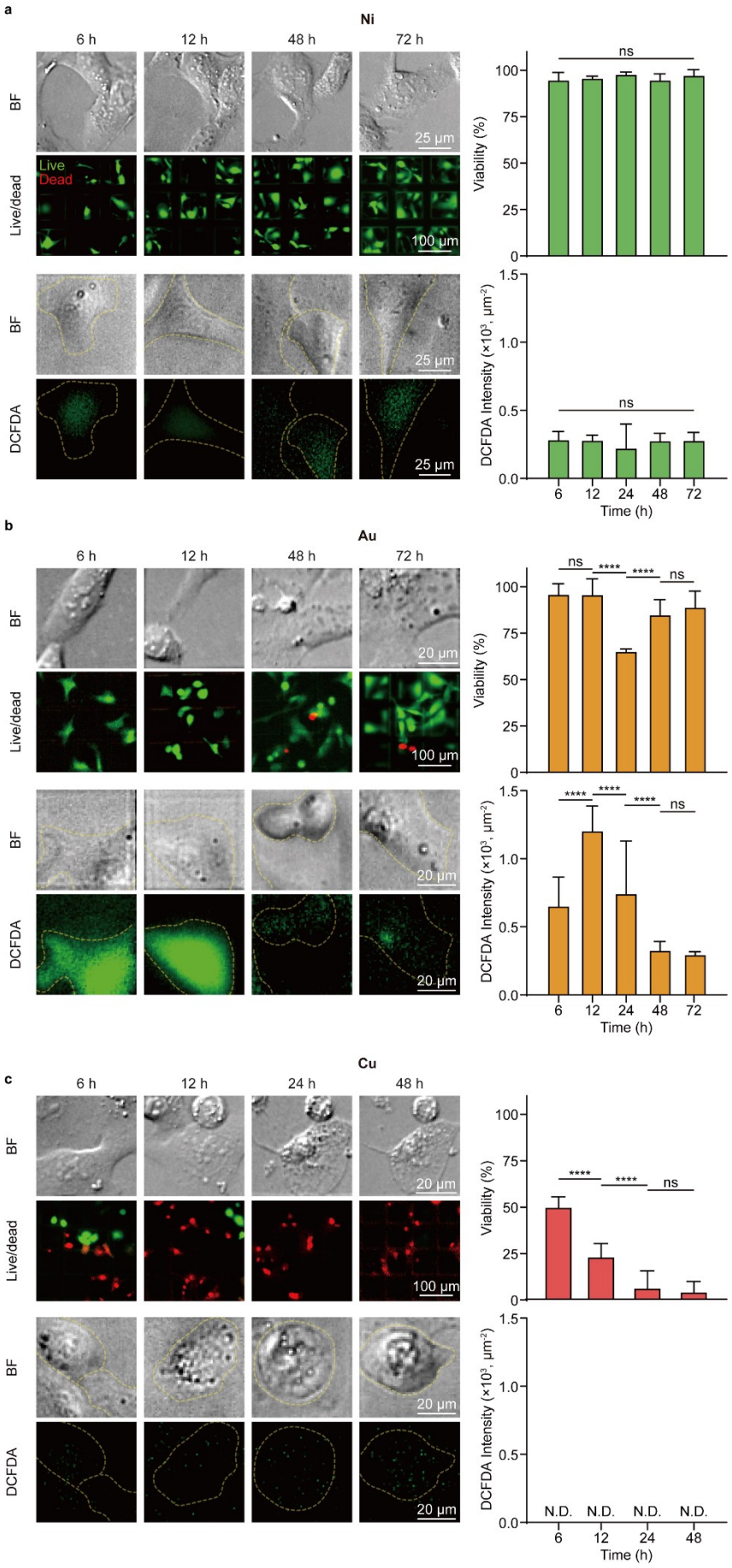
a
Ni
6 h
12 h
48 h
72 h
BF
Live/dead
Live
Dead
DCFDA
25 μm
100 μm
ns
Viability (%)
DCFDA Intensity (×10³, μm⁻²)
Time (h)
b
Au
20 μm
****
c
Cu
24 h
N.D.

Fig. S5. Time-dependent analysis of cell viability and oxidative stress on EM grids. U2OS cells were cultured on (a) Ni, (b) Au, and (c) Cu EM grids and observed using optical microscopy at 6-hour intervals over a 72-hour period. Cell viability and oxidative stress was measured via live/dead assay (green: live cell, red: dead cell) and DCFDA assay (green: oxidative stress), respectively. Ni EM grids supported high cell viability even during long-term culture, with negligible oxidative stress observed throughout the period. In contrast, Au EM grids maintained high viability up to 12 h, which then decline sharply at 24 h. Interestingly, oxidative stress exhibited an inverse trend, peaking at 12 h and gradually decreasing to negligible level at 48 h. This suggest that redox reaction on the Au EM grid were initially active in the culture medium, promoting ROS generation, but later subsided as the reaction reached completion. Cu EM girds showed less than 50% viability from the outset, followed by complete cell death over time. At 48 h, damage to the Cu EM grid made further observation difficult, and DCFDA measurement was not possible due to the absence of live cells.

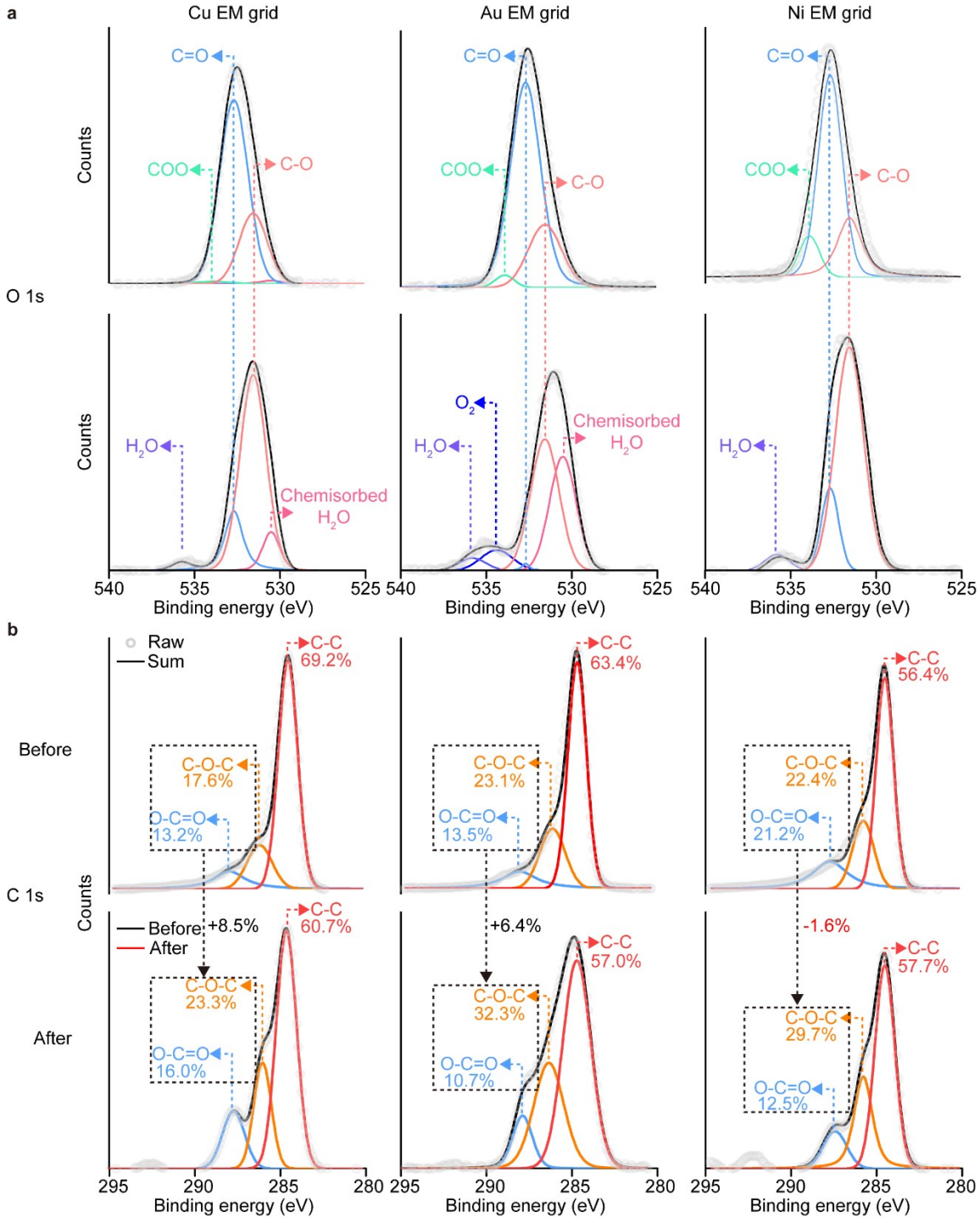

Fig. S6. XPS analysis of EM grids before and after incubation under culture conditions without cells. (a) O 1s spectra before (top) and after (bottom) incubation of Cu (left), Au (middle), and Ni (right) EM grids, demonstrating contributions from C=O (blue), COO- (green), and chemisorbed H_2O (red). Alterations in binding energy suggest oxidation and charge transfer effects on Cu and Au grids, whereas Ni remains stable. (b) C 1s spectra before (top) and after (bottom) incubation, illustrating peaks for C-C (red), C-O-C (orange), and O=C-O functional groups. In Cu and Au EM grids, an increase in oxygen-containing species was observed, indicating chemical modifications. In contrast, Ni EM grids exhibit minimal changes, suggesting higher chemical stability.

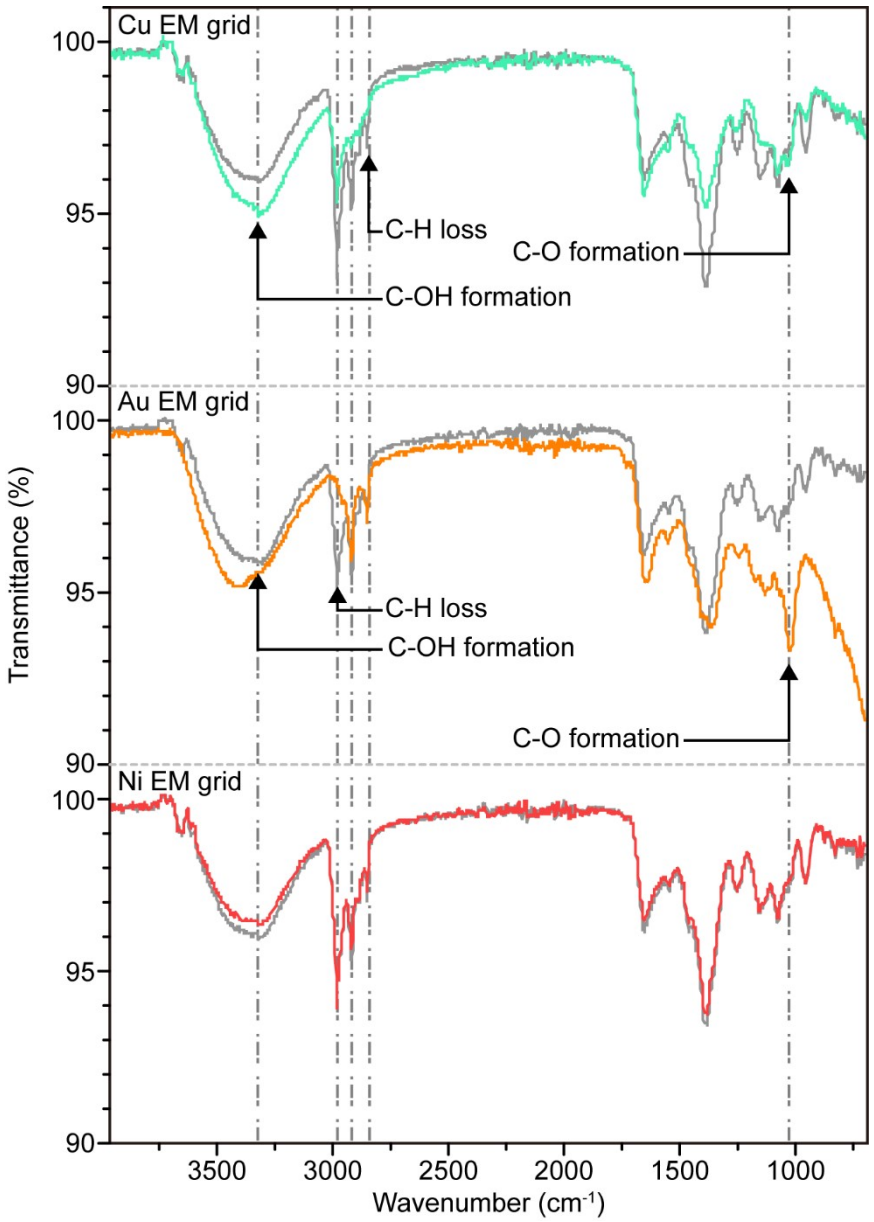

Fig. S7. FTIR spectra of EM grids before and after incubation under culture conditions. FTIR transmittance spectra of Cu (top, green), Au (middle, orange), and Ni (bottom, red) EM grids. The gray lines represent the initial states of each EM grid before incubation, while the colored lines show the spectra after incubation. Significant changes in functional groups are observed, including C-OH, C=O, C-H, and C-O-C, indicating oxidation and chemical modifications in Cu and Au EM grids, whereas Ni EM grids remain relatively stable, demonstrating their superior chemical resistance.

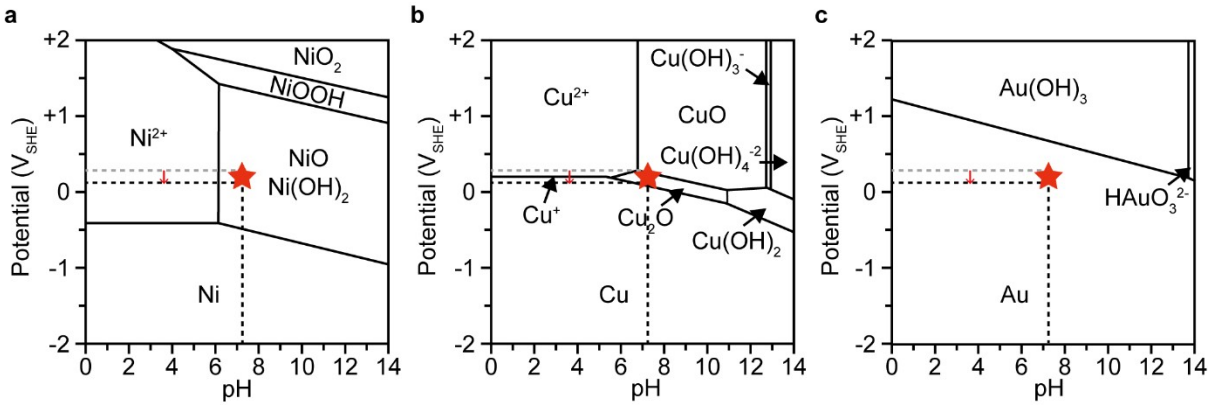

Fig. S8. Thermodynamically stable states of Ni, Cu, and Au under biological environments. Pourbaix diagrams for (a) Ni-H_2O, (b) Cu-H_2O, and (c) Au-H_2O systems. Red stars indicate the approximate pH 7.4 and oxidation-reduction potential (ORP, +230 to +355 mV_{SHE}) conditions typical of the DMEM culture environment. The gray dashed line represents the initial ORP of fresh DMEM, +355 mV_{SHE}, while the black dashed line represents the lower ORP, +230 mV_{SHE} after metabolic activity during cell culture. Under these conditions, $NiO/Ni(OH)_2$, Cu_2O, and Au^0 are thermodynamically favored, consistent with XPS results showing reduction from CuO to Cu_2O and $Au^{\delta+}$ to Au^0, and the passivation behavior of Ni grids.

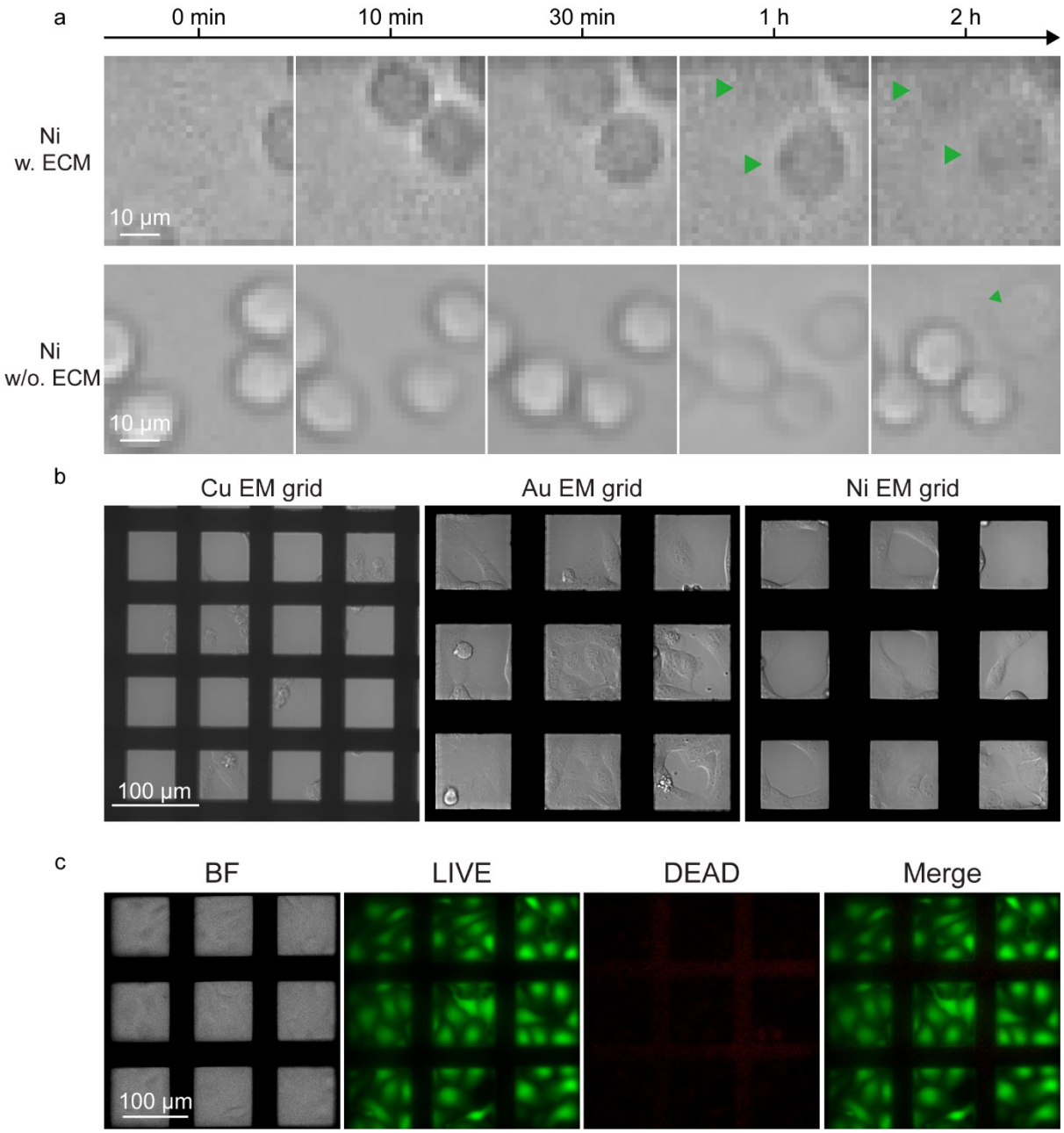

Fig. S9. Cell adhesion and viability on ECM-free Ni EM grids. (a) Time-lapse bright-field microscopy images of U2OS cells cultured on Ni EM grids with (top) and without (bottom) ECM coating. Green arrowheads indicate cell spreading and adhesion over time. The ECM-free condition results in delayed adhesion. (b) Bright-field images of U2OS cells on Cu, Au, and Ni EM grids. Red, yellow, and green dashed boxes indicate abnormal, intermediate, and healthy cell morphology, respectively. (c) Live/Dead assay demonstrates high viability of U2OS cells cultured on ECM-free Ni EM grids.