

SUPPORTING INFORMATION

A Bilayered Nanoshell for Durable Protection of Single Yeast Cells Against Multiple, Simultaneous Hostile Stimuli

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1. Materials.

Gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$)(99%), sodium citrate dihydrate (99%), sodium borohydride (99%), L-cysteine (97%), L-aspartic acid (98%), L-lysine (98%), glycerol (99%), lyticase from *Arthrobacter luteus* ($\geq 2,000$ units mg^{-1} protein), peptone from animal tissue, agar, D-(+) glucose, poly(diallyldimethylammonium chloride) solution (PDDA) (20 wt% in water), poly(sodium 4-styrenesulfonate) (MW~70,000)(PSS), glutaraldehyde solution (50 wt%) were purchased from Sigma-Aldrich Co. (USA). Sodium metasilicate (98%) and H ion exchange resin (Amberlite™ IR-120(H)) were purchased from Alfa Aesar. Tris-EDTA buffer (pH 8.0) and yeast extract were purchased from Sangon Biotech (Shanghai) Co., Ltd. (China). Dimethyl sulfoxide (DMSO), acetone, potassium hydroxide (KOH), sodium hydroxide (NaOH) and hydrogen chloride (HCl), sodium phosphate dodecahydrate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$), sodium chloride (NaCl), iron(III) chloride hexahydrate, iron(II) chloride tetrahydrate, ammonium hydroxide were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Graphite powder (D50<400 nm, 99.95%), 3-aminopropyl triethoxysilane (99%) and hydrazine monohydrate (99%) were obtained from Aladdin Industrial Corporation. LIVE/DEAD® Yeast Viability Kit was purchased from Invitrogen (USA). Syringe filters (0.22 μm) were purchased from Millex.

2. Cell culturing and harvesting.

Freeze-dried *Saccharomyces cerevisiae* was purchased from the China Center of Industrial Culture Collections and grown on yeast-extract-peptone-dextrose (YPD) solid agar plates, containing yeast extract, glucose and peptone in a weight ratio of 1:2:1. A single colony was streaked off the agar plate and used to inoculate YPD broth at 30°C for 24 h. When the optical density (OD) at 600 nm had reached 1 (5×10^7 cells mL), yeast were harvested by centrifugation process (3500 rpm for 10 min) and resuspended in phosphate buffered saline (PBS) (100 mmol L^{-1} , NaCl 150 mmol L^{-1} , pH 7.2) for further experiments.

3. Preparation of yeast cells encapsulated within bilayered nanoshells.

First, L-cysteine powder was added to 90 mL of a gold nanoparticle (diameter 2-3 nm) suspension in distilled water under gentle stirring, resulting in color change from wine red to dark purple. After 30 min, the biohybrid sol was collected by centrifugation and washed with water for 3 times and resuspended in PBS). Yeast cells were then immersed and shaken gently in the biohybrid sol for 5 min to formation of the biohybrid inner layer. Yeast cells were subsequently washed by centrifugation and resuspended in PBS.

Next, the silica outer layer was prepared by sol-gel processing. A metastable silicic acid sol was obtained by mixing ice-cold sodium silicate solution (1.5 mol L^{-1}) with an acid resin, previously rinsed with hydrochloric acid (pH 2.0). The resulting silica sol was separated by filtration and then added into the PBS suspension containing cells encapsulated in the biohybrid layer. Self-assembly of amorphous silica on the yeast surface was initiated by the addition of potassium hydroxide (0.2 mol L^{-1}) to adjust pH value from acidic to neutral. Yeast encapsulated in thus formed bilayered nanoshells were washed by centrifugation and suspended in sterilized PBS for subsequent experiments.

4. Characterization of encapsulated cells.

SEM and an energy-dispersive X-ray spectroscopy (EDX) were conducted (S-4800, HITACHI, Japan) to visualize yeast cell morphology and element distribution of the nanoshells encapsulating the cells, respectively. Encapsulated cells were dropped onto the specimen stage and dried for 30 min at 37°C . SEM was done at an accelerating voltage of 10 kV. EDX was performed at 30 kV in the line scan mode with an Amp time of $25.6 \mu\text{s}$. Fig. S1 shows SEM of sequential states of a single *S. cerevisiae* cell during encapsulation.

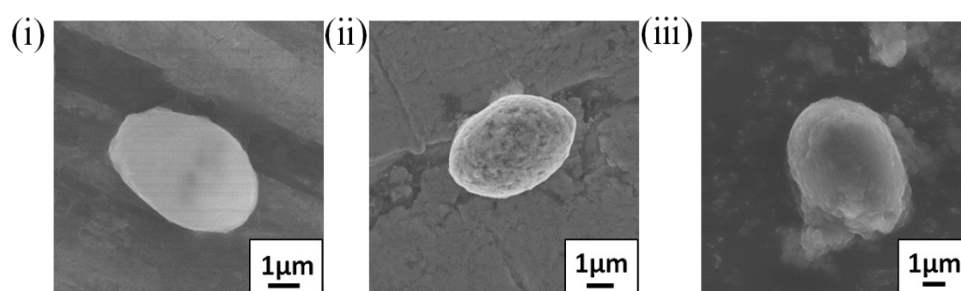


Figure S1. SEM micrographs of the sequential states of a single *S. cerevisiae* cell during encapsulation with a bilayered nanoshell. (i) native yeast cell, (ii) yeast cell with a biohybrid layer containing L-cysteine-coated gold nanoparticles, (iii) yeast cell with a bilayered nanoshell, the outermost surface of which was composed of silica.

For TEM, ultrathin cross-sections were made of the native and encapsulated cells. First, cells were fixed with glutaraldehyde (0.5 vol%) and osmiumtetroxide (1 vol%) after which cells were dehydrated by acetone-ethanol serial washing. After dehydration, cells were embedded in Epon 812/Araldite M resin and the cross-sections with a thickness of 80 nm were made using an ultramicrotome (ULTRACUT UCT, Leica, Germany). TEM was done with an accelerating voltage of 80 kV (Tecnai, Eindhoven, The Netherlands).

For thermogravimetric analysis (LABSYS, Setaram, France), suspended cells were freeze-dried and heated at a rate of $10^{\circ}\text{C min}^{-1}$ to 600°C to evaluate the amount of L-cysteine molecules in the biohybrid layer. The profile of the weight loss as a function of temperature obtained with the thermogravimetric is shown in Fig. S2.

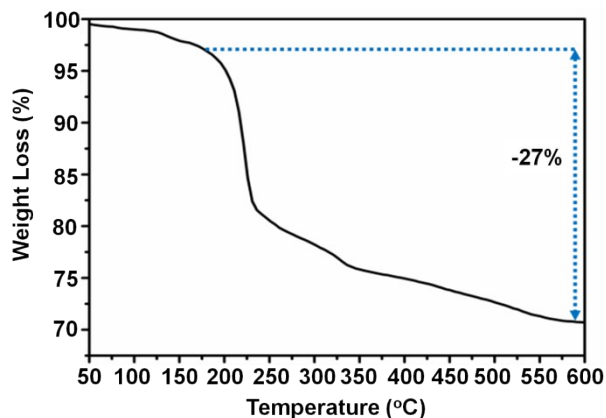


Figure S2. Weight loss as a function of temperature obtained using thermogravimetric analysis on gold nanoparticles/L-cysteine biohybrids.

5. Solid-state ^{13}C NMR and characterization of cell walls.

For use in NMR, cell walls were extracted from whole yeasts. Yeast cells were washed in ice-cold water after harvesting and broken using a high pressure cell craker for 5 times at a temperature just above 0°C in order to prevent endogenous degradation. Cell cracking was evaluated by visual inspection with an optical microscope. Cell walls obtained were washed in cold water by centrifugation for 5 times (1500 rpm, 10 min) and sedimented and subsequently washed in a series of NaCl solutions with decreasing NaCl concentration at 5 wt%, 2 wt% and 1 wt%, successively for two times each) and cold distilled water again for 10 times. Subsequently, NMR spectra were recorded using a NMR spectrometer (500 MZ, Bruker, Germany). High-resolution ^{13}C solid-state NMR was carried out on the cell walls at 500 MHz for ^{13}C , using a Bruker spectrometer. Extracted cell walls were packed as freeze-dried powders. Fig. S3 shows an optical and TEM micrograph of cell walls thus obtained, indicating absence of intracellular materials.

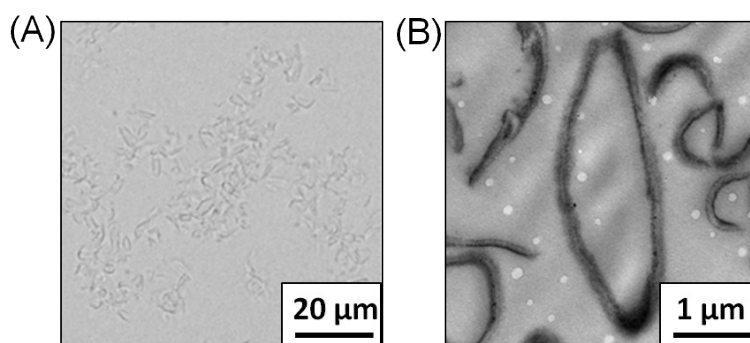


Figure S3. Micrographs of extracted *S. cerevisiae* cell walls. **(A)** Optical micrograph, **(B)** Ultrathin section TEM micrograph of yeast cell wall extracted from yeast cells, showing absence of any intracellular material after cell wall extraction.

6. Zeta potential measurements.

Electrophoretic mobilities were measured in ten-fold diluted PBS with a Zeta sizer Nano Series (ZEN 3600, Malvern) at 25°C using automatic voltage selection. To this end, 1 mL of a yeast cell (with or without encapsulation) suspension, biohybrid solution or silica sol was added to the electrophoresis chamber, while electrophoretic mobilities were converted to zeta potentials using the Smoluchowski equation. The zeta potentials of *S. cerevisiae* in different stages, of biohybrids (L-cysteine-coated gold nanoparticles) and silica are shown in the Table S1.

Table S1. Zeta potentials of native *S. cerevisiae*, cells with a biohybrid layer, cells encapsulated in a bilayered nanoshell, biohybrids and silica sols in ten-fold diluted PBS. Data represent averages with standard deviation over three separate measurements.

Sample	Native cells	Cells with biohybrid layer	Cells in bilayered nanoshell	Biohybrids	Silica sol
Zeta potential (mV)	-16.1±0.5	-18.1±0.7	-19.5±0.9	-19.0±0.6	-20.8±1.4

7. N₂ adsorption/desorption.

N₂ adsorption/desorption isotherms were measured at liquid nitrogen temperature using a Micromeritics TriStar II 3020M system and analysed using the Barrett-Joyner-Halenda model to obtain pore size distribution, surface area (486 m² g⁻¹) and pore volume (0.58 m³ g⁻¹). Prior to N₂ adsorption/desorption, the freeze-dried samples containing cells within bilayered

nanoshells (0.29 g) were transferred to glass tubes which were fixed in the instrument and connected to a nitrogen cylinder for automated pressure increase/decrease. Samples were heated to 350°C to remove the organic molecules and degassed under vacuum for 20 h at 200°C. Nanoshell surface area and pore size distribution were calculated from the adsorption branch of the isotherm according to Brunauer-Emmett-Teller and Barrett-Joyner-Halenda, respectively.

8. Application of single and simultaneously-acting, multiple hostile stimuli.

Lyticase-tolerance. Freeze-dried lyticase (3.8 mg) was dissolved in a mixture (1 mL) of glycerol and TRIS-EDTA (TE) buffer (1:1 vol:vol). 10 µL of the lyticase solution was added to 1 mL of native or encapsulated yeast cell suspensions at 37°C for 5 h. Aliquots were withdrawn every 5 min to determine the numbers of viable yeast (see below).

Thermo-resistance. For application of a high temperature stimulus, yeast cell suspensions (1 mL) in glass tubes were kept in an incubator at a preset temperature of 40°C. Aliquots were withdrawn every 5 min to determine the numbers of viable yeast cells (see below). Cell morphology was observed after 12 hour incubation using SEM (see above). For cell surface temperature measurements, freeze-dried cells were kept in glass tubes in a temperature-controlled water bath at temperatures between 35°C and 55°C. Cell surface temperature was measured by using a high-sensitive thermometer.

UV light exposure. Native or encapsulated yeast cell suspensions (5 mL in PBS) were exposed to UV light (15 W) for 5 h without capping. Aliquots were withdrawn every 5 min to determine the numbers of viable yeast cells (see below).

Multiple, simultaneously-acting stimuli. All stimuli described above, were also applied in concert.

Recycling of exposed yeast. Yeast cells after application of single or simultaneously-acting, hostile stimuli were washed in sterilized PBS by centrifugation at 10,000 rpm for 2 h (counting as one cycle). After each cycle, yeast were resuspended in PBS and exposed again to a hostile stimulus up to 10 cycles. The numbers of viable yeast cells were determined after each cycle (see below).

Cell viability assays. Aliquots of yeast cells taken during exposure to hostile stimuli were collected by centrifugation, washed with PBS, serially diluted and streaked on YPD agar plates. The number of colony forming units (CFUs) was enumerated from agar plates with serial dilutions yielding between 30-300 CFU. Viability was presented as a percentage number of CFUs found relative to the initial viabilities recorded by counting numbers of colonies. To

mitigate biological variability, three replicates were performed with separately cultured yeast and independently encapsulated cells.

Yeast cell viability was also determined using LIVE/DEAD[®] Yeast Viability Kit staining. The FUN[®] 1 stain is hydrolyzed to a red-yellowish fluorescent molecule in living, metabolically active cells with an intact plasmatic membrane, while dead cells appear green-yellow fluorescent upon excitation at 480 nm. A FUN 1 stock solution ($20 \mu\text{mol L}^{-1}$) was prepared in DMSO and $20 \mu\text{L}$ of FUN 1 solution ($20 \mu\text{mol L}^{-1}$) was mixed with an aliquot ($20 \mu\text{L}$) of yeast cell suspensions after application of hostile stimuli and incubated at 30°C in the dark for 30 min. Fluorescence micrographs were captured using a fluorescence microscope (Olympus, Japan) and spectra recorded on a UV-visible spectrophotometer (UV-2550 SHIMADZU, Japan).

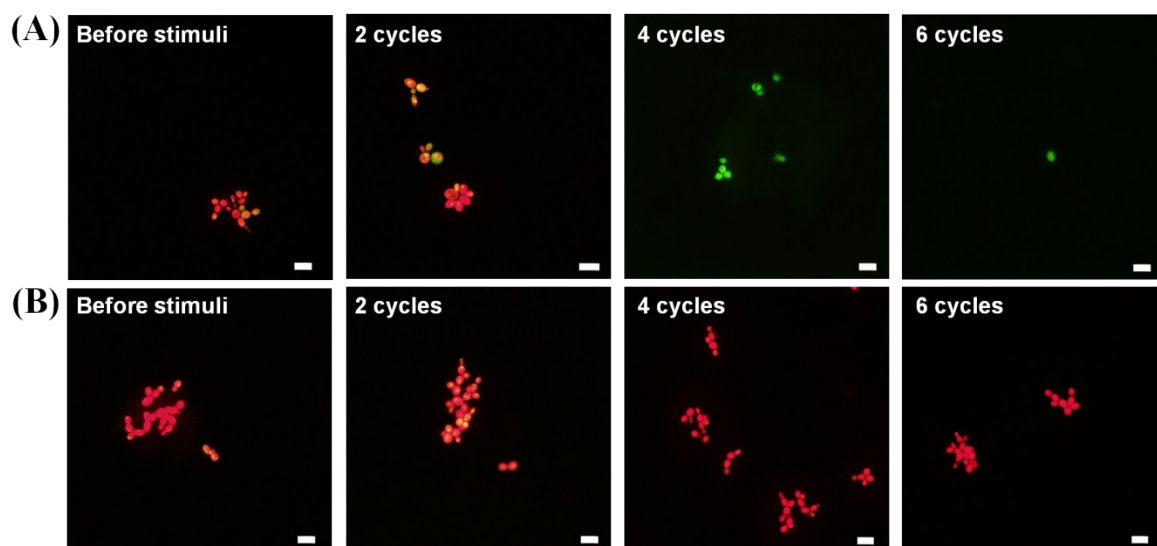


Figure S4. Fluorescence images of FUN1 (LIVE/DEAD[®] Yeast Viability Kit, Invitrogen) stained *S. cerevisiae* cells exposed to multiple, simultaneously-acting stimuli. Live cells show red fluorescence, while dead cells show green fluorescence emission. (A) Native yeast cells without encapsulation before and after multiple cycles of simultaneous hostile stimuli with lyticase, high temperature (40°C) and UV light exposure (B) Same as panel A, now for yeast cells encapsulated in bilayered nanoshells. Scale bars equal $10 \mu\text{m}$.

9. Miscellaneous encapsulation methods applied.

Miscellaneous encapsulation methods were applied for comparison. Briefly, harvested yeast cells were suspended in a PDDA solution (5 mg mL^{-1} in distilled water) or PSS solution (5 mg mL^{-1} in distilled water) for single polyelectrolyte encapsulation. Additionally, cells were

suspended in PDDA and PSS solutions to form a PDDA/PSS layer. Also, PDDA/PSS encapsulated cells were exposed to a PDDA solution for the second time and subsequently suspended in gold nanoparticle suspension. Each deposition step took 5 min. Encapsulated cells were collected and washed with PBS. Silica layer formation on PDDA/PSS/PDDA/gold nanoparticle encapsulated yeast cells was achieved with amorphous silica as described above (Section “*Preparation yeast cells within bilayered nanoshells*”).

To assess potential negative effects of the polyelectrolyte solutions used in the various encapsulations, yeast cells were added in to PBS (control), PDDA, PSS and PDDA/PSS solutions, PDDA/PSS/PDDA/gold nanoparticle suspensions and an L-cysteine/gold nanoparticle biohybrid suspension for 4 h and 8 h. Cell morphologies in different solutions were characterized by SEM for potential changes in morphology.

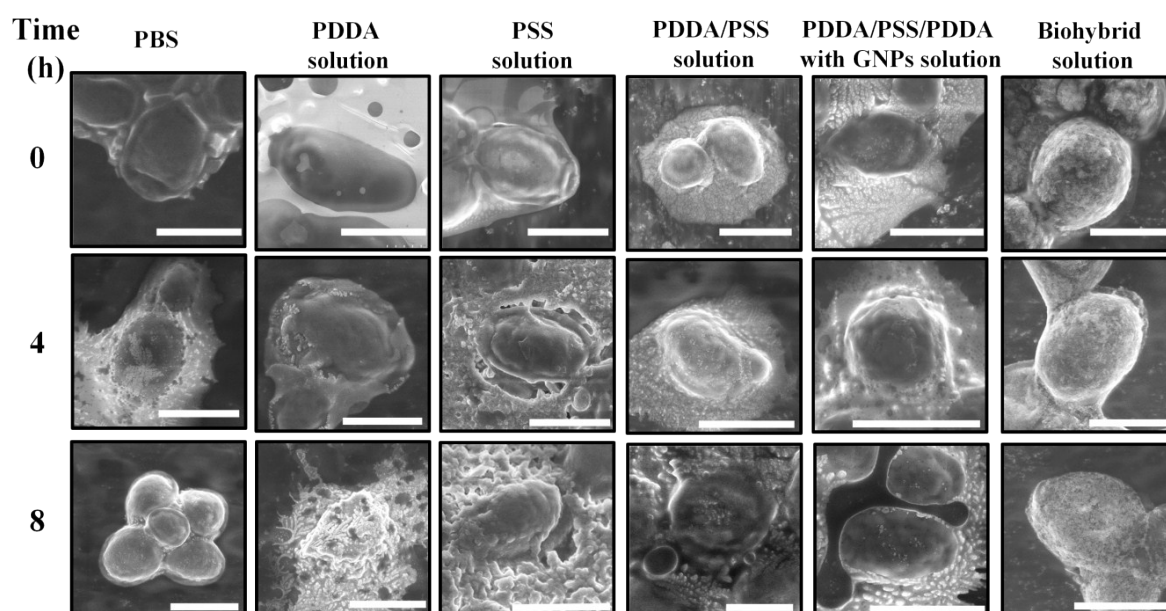


Figure S5. SEM images of the morphologies of *S. cerevisiae* suspended in different polyelectrolyte solutions involved in encapsulation: PBS, PDDA solution, PSS solution, PDDA/PSS solution, PDDA/PSS/PDDA with gold nanoparticles (GNPs), and biohybrid solution containing L-cysteine with GNPs. Scale bar equals 5 μm . The original morphologies of cells were maintained when cells were suspended in the biohybrid solution up to 8 h, while cells in other polyelectrolyte solutions could not maintain their morphology after 4-8 h.

10. Lyticase adsorption by a biohybrid sol and amorphous silica.

Lyticase solution (0.1 mL) was added into 2 mL of a biohybrid solution or amorphous silica sol. The adsorbed lyticase by biohybrids and silica was removed by centrifugation (5000 rpm, 15 min). The concentration of residual lyticase in the solution was evaluated using a standard Bradford protein assay after 1 h, taking the initial lyticase concentration at time 0 as 100%.

Table S2. Adsorption of lyticase to the biohybrid and amorphous silica.

Samples	Relative lyticase concentration at time 0	Relative lyticase concentration after 1 h
Biohybrid	100%	56%
Silica	100%	82%

11. Yeast cells encapsulated within bilayered nanoshells using other amino-acid molecules.

L-aspartic acid (0.15 g) powder was added to the gold nanoparticle suspension (30 mL) in distilled water under gentle stirring. L-lysine powder (0.15 g) was dissolved in distilled water (10 mL), followed by mixing with gold nanoparticle suspension (30 mL). The biohybrid sol was collected by centrifugation. Yeast cell suspension was immersed with biohybrid sol for 5 min. The outer silica layer was prepared by sol-gel processing which has been described in section “*Preparation yeast cells within bilayered nanoshells*”. Figure S6 shows SEM micrographs of bilayered nanoshell encapsulated cells using aspartic acid and lysine molecules and their viability after different cycles of simultaneously-acting hostile stimuli.

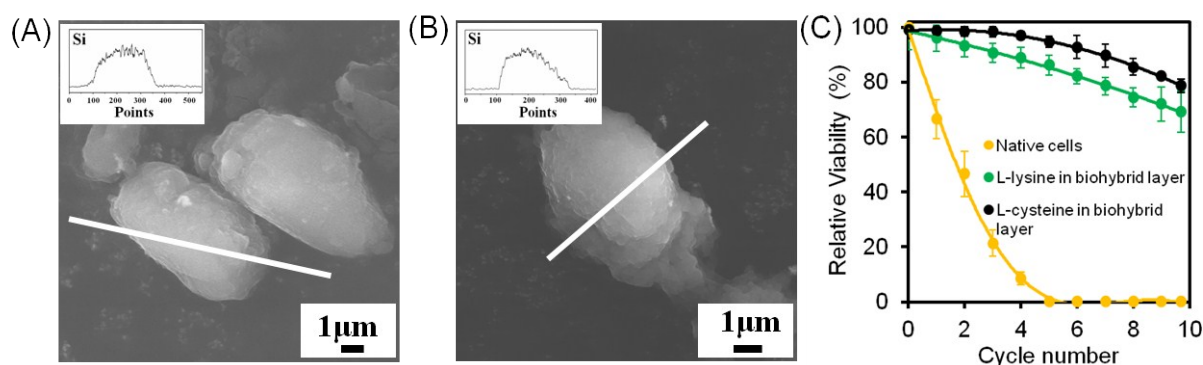


Figure S6. Potential use of other amino acids than L-cysteine to create a bilayered nanoshell around *S. cerevisiae*. (A) SEM images and EDX line scan for Si of yeast cells encapsulated in

a bilayered shell with GNPs and amorphous silica using aspartic acid for biohybrid layer formation. Inset shows the EDX line scan for Si of yeast cells encapsulated in a bilayered nanoshell. (B) Same as panel (A), using lysine molecules for biohybrid layer formation. (C) Comparison of the viability of yeast cells encapsulated in bilayered nanoshells using Lysine with the viability of native yeast cells upon simultaneously-acting hostile stimuli.

12. Two-Dimensional Finite-Difference Time Domain (TDFD) simulation of thermal and UV protection.

To calculate the contribution of the biohybrid inner layer and silica outer layer with respect to the attenuation of thermal stress and UV light on a single bilayered nanoshell encapsulated cell, a two-dimensional TDFD method was used.

For simulated thermal protection, a cell was projected on a square grid. Finite differentials were numerically computed for both space and time, taking constant surrounding temperature as a boundary condition. The thickness and diffusion constant for the outer silica layer were defined as 200 nm and $3.4 \times 10^{-7} \text{ mm}^2 \text{ s}^{-1}$, respectively and for the inner biohybrid layer were taken as 70 nm and $1.0 \times 10^{-7} \text{ mm}^2 \text{ s}^{-1}$, respectively. Mathematically, temperature distribution within the encapsulated cell is governed by

$$\frac{\partial T}{\partial t} = D\nabla^2 T \quad (\text{Equation S1})$$

where D represents thermal diffusivity of the layer, and that can be numerically solved (Movie 1).

For simulated UV protection, relevant differential equations were solved using the Yee algorithm, based on the refractive and absorptive properties of the bilayered nanoshell as included in the complex refractive index n_c or permittivity, i.e. the squared complex refractive index $n_c = n + i\kappa$, in which n is the refractive index and κ the permittivity. The electromagnetic field was subsequently computed to obtain a full solution of differential equations governing the electromagnetic field propagation from which the refraction and distance dependent intensity decay was calculated according to the Beer-Lambert law, written as

$$\frac{I_o}{I_i} = 10^{-Ax} = e^{-A \ln(10) x} = e^{-4\pi\kappa x/\lambda}$$

(Equation S2)

where I_o and I_i are the output and input intensities, respectively, A is the absorbance at a distance x , and λ is the wavelength. Accordingly, the UV intensity attenuation due to refraction and absorption can be calculated at various distances x in the medium (see also simulation in Movie 2).

13. Post-functionalization of cells.

A fresh silica sol was added to a yeast suspension in a biohybrid solution, followed by the addition of magnetic Fe_3O_4 nanoparticles or graphene (each at 0.2 mg mL^{-1}). The formation of Fe_3O_4 /silica or graphene/silica layers on the biohybrid inner layer was initiated by adding KOH (0.2 mol L^{-1}) to adjust pH value from acid to neutral. The electrical conductivity of suspensions with yeast encapsulated in bilayered nanoshells with graphene was measured by using a Probe Station (Advanced Research System, Macungie, USA) .