Dynamic Surface Properties of PEG-coated CuS Nanoparticles Alter their Interaction with Cells Revealed by Surface-Enhanced Infrared Spectroscopy

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Experimental Procedures

Materials

Sodium tetrachloroaurate dihydrate (NaAuCl₄·2H₂O) was purchased from Alfa Aesar (Shanghai, China). Graphite powder (99 %), sodium sulfite anhydrous (Na₂SO₃, ACS ≥ 98.0 %), sodium thiosulfate pentahydrate (Na₂S₂O₅·5H₂O, ACS 99.5 %), hydrofluoric acid (HF, GR 40.0 %) and ammonium chloride (NH₄Cl, ACS 99.5 %,) were purchased from Aladdin Ltd. (Shanghai, China). Ammonium fluoride (NH₄F, ACS ≥98.0 %) and p-aminothiophenol (p-ATP) were bought from J&K scientific Ltd. (Beijing, China). Sulfuric acid (H₂SO₄, 95-98.0 %), potassium permanganate (KMnO₄, 99.5 %), hydrogen peroxide (H₂O₂, 30 %), sodium dihydrogen phosphate (NaH₂PO₄·2H₂O, 99%), and sodium chloride (NaCl, 99.5%) were purchased from Beijing chemical company. Sodium nitrate (NaNO₃, 99 %) was supplied by Beijing HLCC fine chemical company. Disodium hydrogen phosphate dodecahydrate (Na₂HPO₄·12H₂O, 99 %) was acquired from Xilong Chemical Co., Ltd. Methoxypoly(ethylene glycol)-thiol (mPEG-SH, MW 5000) was obtained from Shanghai ToYong Bio Co., Ltd (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin (100×), 0.25% trypsin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Beijing Dingguo Biotechnology Co. Standard fetal bovine serum (FBS) was purchased from Tianjin Kang Yuan Biological Manufacture Co., Ltd. (Tianjin, China). Poly-L-lysine (PLL, MW15.000–30.000) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). D-Phenylalanine (Phe) was purchased from Yuanju biotechnology Co., Ltd. (Shanghai, China). 10 mM PBS buffer (pH=7.4) was prepared using NaCl (136 mM), KCl (2.6 mM), KH₂PO₄ (2 mM), and Na₂HPO₄ (8 mM). All solutions were freshly prepared with ultrapure water (18.25 MΩ/cm) generated using a Millipore Milli-Q system (Billerica, MA, USA). All chemicals were used without additional purification.

Apparatus

The UV-Vis absorption spectrum was recorded on Lambda 25 spectrometer (PerkinElmer, USA) with a 1.0
cm optical path length quartz cuvette. Scanning electron microscope (SEM) images combined with energy dispersive X-ray spectroscopy (EDS) spectrum were taken with a XL30 ESEM-FEG field-emission scanning electron microscope (FEL COMPANY, Netherlands). Atomic force microscope (AFM) images were conducted on Multimode-V atomic force microscopy (Veeco Instruments, USA) with a typical tapping-mode measurements in air, and the topography of images were processed by the Scanscope 8 software. The HeLa cells incubated on the surface of rGO-Au/Au film on were recognized according to the contrast to in the optical microscope by Nanoscope 9.3 software. The morphology and size of dried samples were examined using a transmission electron microscope (TEM, Hitachi-600, Tokyo, Japan) at an accelerating voltage of 100 kV with carbon-enhanced copper grids. The corresponding lattice fringes (d-spacing) were evaluated by high resolution transmission electron microscopy (HRTEM, H-600 electron microscope, Hitachi, Japan). The X-ray energy dispersive spectroscopy (EDS) spectra of CuS NPs dried on the Si wafer in air were carried on a XL30 ESEM-FEG field-emission scanning electron microscope (FEL COMPANY, Netherlands). UV-Vis absorption spectra were carried out with a LAMBDA 25 spectrometer (PerkinElmer). Raman spectrum was obtained on micro-Raman spectroscopy system (Renishaw, UK) using (laser wavelength: 532 nm; power: 10 mW; lens: 50×objective; acquisition time: 20 s). X-ray photoelectron spectra (XPS) experiments were performed on a monochromatized Al K Alpha radiation with the pass energy 100.0 eV (ESCALAB 250, USA). The hydrodynamic particle size and Zeta potential of CuS NPs were determined by a dynamic light scattering (DLS, ZEN 3600, Malvern Instrument, UK). The copper content was quantified using inductively coupled plasma mass spectroscopy (ICP-MS, DIONEX ICS-1000, America) with parts per billion sensitivity.

**Synthesis of graphene oxide (GO)**

Graphene oxide (GO) was synthesized according to the modified Hummer’s and Offeman’s method as described everywhere\(^1\). Briefly, graphite (1 g) and 98 % H\(_2\)SO\(_4\) (23 mL) were mixed in a round-bottomed flask and stirred for 24 h, then NaNO\(_3\) (0.5 g) and KMnO\(_4\) (3 g) were slowly added with constant stirring for 30 min.
and 5 min, respectively, in an ice bath. Then the temperature was kept at 35 ºC for 5 min, and 46 mL of distilled water was slowly added and stirred for 40 min. Finally, the flask was transferred to a 98ºC oil bath for 25 min, then 3.4 % H₂O₂ (31 mL) was added to terminate the reaction to get brown yellow solution. The obtained solution was washed and dialyzed against water, then sonicated and centrifuged.

**Preparation of rGO-Au-decorated Au Plasmonic Substrate.**

The Au film decorated with rGO-Au (rGO-Au/Au) was electroless deposited onto the Si prism or wafer, which was polished with Al₂O₃ slurry (1.0 μm) followed by washing thoroughly with deionized water. After immersing in a 40 wt % NH₄F aqueous solution for 3 min, the rGO-Au/Au film was prepared by exposing the treated surface to a 1:1:1 (v/v/v) mixture of 0.3 M Na₂SO₃ + 0.1 M Na₂S₂O₃ + 0.1 M NH₄Cl, 3 % HF, and 1.2 mg/mL NaAuCl₄ aqueous solution mixed with 2.5 mg/mL GO for 4 min at 55 ºC. The obtained substrate was cleaned by electrochemical oxidation–reduction scans in 0.1 M H₂SO₄ solution over the potential range of 0.1–1.4 V versus Ag/AgCl. At last, the rGO-Au/Au film coated Si prism was mounted on a home-built poly(trifluorochloroethylene) cell with a Viton O-ring. In order to obtain the desired enhancement effect for sulfhydryl and aromatic molecules, the NaAuCl₄ aqueous solution mixed with different mass of GO dispersion (mₐu/m₈0=4.8~66) was used to detect p-aminothiophenol (p-ATP) and methylene blue (MB) molecules.

To evaluate the enhancement factor (EF) of rGO-Au/Au plasmonic substrate, a reference spectrum of taking bare Si or rGO-Au/Au-coated Si prism was firstly recorded and then ~0.32 μg (~monolayer thickness)² of p-ATP ethanol solution was dropped on the surface of the bare Si and rGO-Au/Au-coated Si prism, respectively. The sample spectra were recorded until total evaporation of ethanol by averaging 521 scans at a spectral resolution of 4 cm⁻¹.

**Preparation and intracellular uptake of CuS NPs**

The synthesis of CuS NPs in water was prepared according to the literature by using PEG as a stabilizer to
enhance biocompatibility, and prevent nonspecific interactions in physiological environments. In a typical procedure, after the mixing of 25 mL of PEG-SH solution (1 mg/mL) and 25 mL of CuCl₂ (5 mM) aqueous solution, 1 mL of Na₂S (0.25 M) was slowly added dropwise to the solution. The pale-blue CuCl₂ solution turned dark-brown immediately. After 10 min, the reaction flask was transferred to an oil bath (~90 ºC) with continuous stirring for 10 min until a dark-green solution was obtained. At last, the flask was cooled in an ice-water bath to terminate the reaction. The as-synthesized PEG-coated CuS NPs was dialyzed with molecular weight cut-off (MWCO) of 30 kDa and stored at 4 ºC for later use.

To more directly explore the endocytic mechanism of CuS-PEG NPs internalized into HeLa cells, cells were pretreated with various inhibitors including dynasore (30 µg/mL, dynamin inhibitor), chlorpromazine (CPZ, 10 µg/mL, clathrin inhibitor), filipin (10 µg/mL, caveolae inhibitor), and Cyt D (1 µmol/L, inhibitor of actin polymerization related phagocytosis and clathrin/caveolin independent endocytosis) for 1 h at 25°C or 4°C (energy independent pathway) detected using ICP-MS and ATR-SEIRS. Then, the cells were washed with PBS three times and cultured with CuS-PEG NPs at the concentration of 100 µg/mL for 2 h, the process of cell changes was monitored by ATR-SEIRS in real-time. And then cells were washed with PBS for three times, trypsinized, resuspended, counted, and centrifuged down, and the cell pellet was digested in 1 mL of aqua regia (V₇HCl:V₇HNO₃=3:1). Cell concentrations were determined by hemacytometry and the intracellular copper content was quantified using ICP-MS with parts per billion sensitivity. In control, cells were placed in 1 mL PBS without nanoparticles.

Immobilization of Cells on rGO-Au/Au Film

Before culturing HeLa cells, the home-made poly(trifluorochloroethylene) cell and necessary experimental tools were sterilized by 70% ethanol and UV irradiation. 2 mL of cell suspension (5×10⁵ cell/mL) was seeded on the poly-L-lysine layer (0.1 mg/mL PLL) modified rGO-Au/Au film. After incubation for 24 h, the IR cell was mounted on SEIR spectrometer. After washing three times with PBS, cells were exposed to 100 µg/mL of
PEG-CuS NPs by adding 25 µL PEG-CuS NPs aqueous solution (4 mg/mL) into a volume of 1 mL of PBS.\textsuperscript{6} CuS NPs induced SEIRA spectra were obtained by taking cells in PBS as a reference spectrum. After incubation with 100 µg/mL PEG-CuS NPs for 2 h, the cells were washed three times with PBS and followed by being irradiated for 20 min using the 980 nm diode laser with power of 1 and 1.5 W/cm\textsuperscript{2}. As the control experiments, 25 µL H\textsubscript{2}O, supernatant of CuS NPs (20 day) and 5 mM CuCl\textsubscript{2} were used to replace CuS NPs and added into the ATR chambers. Unless noted specifically, the control experiments were performed following the same protocol and each measurement was repeated in triplicate.

**Cell culture and viability test**

Human epithelial cervical cancer cells (HeLa) were routinely cultured in the fresh Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin in a humidified incubator containing 5% CO\textsubscript{2} at 37 ºC. Media were changed every third day. For subculture, the cells were washed three times with PBS (10 mM, pH=7.4) and incubated with trypsin solution (0.25 %) for 3 ~4 min at 37 ºC to detach the cells. Complete media were then added in the flask at room temperature to inhibit the effect of trypsin.

The biocompatibility and cytotoxicity of the CuS NPs was determined by the methyl thiazolyl tetrazolium (MTT) assay. In brief, HeLa cells was seeded into a 96-well microtiter plate with a density of 1×10\textsuperscript{4} cells per well and then cultured in 5% CO\textsubscript{2} at 37 ºC. After 48 h, the HeLa cells were treated with PBS for 2 h and 6 h, and exposed to CuS NPs at different concentrations (80, 100 and 200 µg/mL) for 2 h. After washing three times with PBS, 200 µL of MTT reagent (0.5 mg/mL MTT in PBS, pH 7.4) was added into each well. Then cells were incubated for another 4 h and the solution was suctioned off carefully. 100 µL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the purple formazan crystal produced by active mitochondria of proliferating cells. The absorbance at 550 nm was measured with a Synergy HT Multi-mode Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The percentage of cell viability was calculated as follows:
After the FTIR experiment, cell viability on the rGO-Au/Au substrate was roughly assessed using top-view microscope for cell monolayer integrity and morphology, and the trypan blue exclusion test to stain dead cells. Cells were trypsinized and resuspended in trypan blue (0.4 %, g/ml) for 3 min. Cell viability was calculated under 10×magnification using a hematocytometer. The percentage of cell viability was calculated as follows:

\[
\frac{N_{\text{viable cells}}}{N_{\text{total cells}}} \times 100\%.
\]

**Simulation**

To simplify, the ellipsoid gold nanoparticle (R₁=30 nm, R₂=10 nm) obtained from AFM image and a rGO layer of 4 nm thick inferred from Raman data were considered, and simulation was performed by using 3D finite-difference time-domain (FDTD) method on the basis of time varying electromagnetic Maxwell’s equations.⁷, ⁸ The incident light with a wavelength range from 2.5 to 10 µm propagating along the z direction was normally illuminated on the surface. Periodic boundary conditions and perfectly matched layer conditions were used to evaluate near-field enhancement data \(|E/E_0|\) of rGO-Au/Au film. The optical permittivity parameters of Au and rGO used in simulation were extracted from the data of Palik and Hwang.⁹, ¹⁰

**Plasmon Nano-imaging**

The scattering-type scanning near-field optical microscopy (s-SNOM, Molecular Vista) used for this work was based on an AFM probe (a gold coated Si cantilever, 70 nm tapping amplitude and 270 kHz resonance frequency), which was illuminated by a focused infrared light from pulsed quantum cascade laser (QCL, Block Engineering). The plasmon nano-imaging of sample could be obtained simultaneously via mapping the topography and the near-field scattered intensity detected by a liquid-nitrogen-cooled MCT detector. In order to suppress near field contribution from the tip-sample region, a versatile “Generalized Lock-In Amplifier” (GLIA) approach, which calculates the amplitude and phase of the scattered field, was used in the first harmonic.², ¹¹
Supplementary Figures

Figure S1

**Figure S1. Preparation and characterization of GO.** (A) Schematic of chemical preparation of GO. (B) TEM image of GO. (C) AFM image of GO in tapping mode and the corresponding height profile. (D) Zeta potential of GO. (E) UV-vis absorbance spectrum of GO aqueous solution. (F) XPS survey spectrum of GO. (G) ATR-FTIR absorption spectrum of GO was dried by N₂.

A schematic illustration of the procedure for fabricating GO using modified Hummer’s method was shown in Figure S1. The surface structure and morphology of GO were further confirmed by TEM and AFM, the size of the synthesized GO is relatively uniform (~400 nm) and the height profile is ~1 nm thickness, corresponding to
a typical thickness of a two-dimensional single-layer GO sheet. The UV-vis spectrum exhibited a characteristic absorption peak at 230 nm and a shoulder peak at 300 nm, attributing to the electronic transition of $\pi-\pi^*$ of C=C bonds and n-$\pi^*$ of C=O bands, respectively.\textsuperscript{12} The oxygen content of as-prepared GO was $\sim$31\% based on XPS measurement. The ATR-FTIR spectrum of GO showed that the $\nu$ O-H stretching vibration (a broad 3450 cm$^{-1}$), $\nu$ C=O stretching of -COOH or C=O groups (1739 cm$^{-1}$), the resonance at 1622 cm$^{-1}$ can be assigned to the bending vibrations of the adsorbed water molecules, and it contains a hidden 1600 cm$^{-1}$ peak from skeletal vibrations of unoxidized graphitic domains, $\nu$ C-OH (1363 cm$^{-1}$), $\nu$ C-O-C (1227 cm$^{-1}$) and $\nu$ C-O (1067 cm$^{-1}$), respectively, the corresponding position of absorption bands could be affected by structure water and free water.\textsuperscript{13-16} Due to lots of surface oxygenic functional groups, $\zeta$ potential change of GO was about -30 mV.
**Figure S2**

![Cell Viability Test](image)

**Figure S2. Cell viability test.** (A) MTT viability assay of the cells treated with PBS for 2 h and 6 h, CuS-PEG NPs at various concentrations (80, 100, and 200 µg mL$^{-1}$) in PBS for 2 h. (B) The image of trypan blue exclusion test of CuS NPs incubated cells which were trypsinized from the rGO-Au/Au substrate.

As shown in Figure S4, cells treated with CuS NPs still maintain good activity and biocompatibility validated by cell viability experiments of MTT and trypan blue exclusion test.
Figure S3. The structures of CuS NPs over time. (A-B) TEM with corresponding diameter distribution images, (C-D) High-resolution transmission electron microscope (HRTEM) images, (E-F) X-ray energy dispersive spectroscopy (EDS) spectra, (G) Optical absorption spectra at the concentration of 400 \( \mu \text{g/mL} \), (H) FTIR spectra and (I) XPS C1s spectra of CuS-new NPs and CuS-old NPs.

The morphology of the air-dried CuS-new NPs and CuS-old NPs were observed by TEM images with an average size of \(~10\) nm (Figure S3A) and \(~8\) nm (Figure S3B), respectively. Then their crystal structure were characterized by high resolution transmission electron microscopy (HRTEM) as shown in Figure S3C-D, which revealed the hexagonal CuS nanostructures with d-spacing of \(~0.301\) nm assigned to (102) planes and \(~0.28\) nm.
assigned to (103) planes$^{3,17}$. The energy-dispersive spectroscopy (EDS) spectra clearly showed that the coexistence of C, O, S and Cu elements (The Si element come from the substrate of Si wafer) and the ratio of Cu:S was approximately 1:1. The morphology and structure of the CuS NPs have not changed substantially, however, by ICP results, we can see that a small amount of copper ions (4 µg/mL) dissociated from the CuS NPs, causing the UV-Vis-NIR absorption peak intensity of the CuS NPs to decrease and the maximum absorption peak was blue-shifted from 1018 to 952 nm (Figure S3G) due to the effect of quantum size confinement$^{3}$, and the band of $\nu$(Cu-O) was appeared in the infrared spectrum of CuS-old NPs group, suggesting that the coordination between the dissociated copper ions and PEG. In order to verify this, we further use XPS technique, a useful sample surface element state analysis tool, to detect different period of CuS NPs. As we can see, the more prominent satellite peaks for CuS-old NPs at around 944.2 eV and 962.6 eV demonstrate the existence of Cu (II) in the paramagnetic chemical state on the surface of CuS NPs,$^{18}$ further indicating that there are more Cu$^{2+}$ species existed on the surface of PEG-CuS NPs.
The prepared HeLa cells were collected by centrifugation (1000 rpm, 10 min) and washed four times with 0.9 % NaCl solution to remove growing medium. A total of 50 μL of the resulting cell pellet was introduced onto the surface of Si crystal, and ATR-FTIR spectra of HeLa cells were collected after dried by ultrapure nitrogen flux to remove any excess water and by taking the bare Si as a background (Figure S4). In Figure S4, we can see that this situation should mainly come from the influence of ν(N-H) band, while it isn’t same with our spectra, in which the absorption peaks of 3250 cm⁻¹ and 3680 cm⁻¹ appear simultaneously (Fig. 3). In turn, this can prove that our absorption peaks should mainly come from interfacial water interference.
Figure S5. Gaussian curve-fitting spectra between 1645 and 1490 cm\(^{-1}\) with experimental (black line) and fitted curves (red line) including deconvoluted components in the course of the interaction of CuS-old NPs and HeLa cells.

As we can see, the peak range between 1645 and 1490 cm\(^{-1}\) during the interaction of CuS-old NPs and HeLa cells was very board, so the movement of the maximum peak position may be a change in different chemical bonds. Therefore, the Gaussian curve-fitting analysis was carried out by using the OPUS 7.5 software in the interactive mode. Figure S5 shows that three deconvoluted components at ~1600, 1560 and 1543 cm\(^{-1}\), which are attributed to $\delta$(N-H) or $\nu_{\text{asym}}$(COO\textsuperscript{−}) and amide II, respectively\textsuperscript{19-21}. The absorption peaks of carboxyl and amino groups are derived from the coordination of copper ions. As time goes on, the coordination effect increases gradually. So, the shift of the peaks represents the gradual coordination of copper.
Figure S6

Figure S6. Interaction of CuS-old NPs and HeLa Cells assessed by SEIRA spectra with and without inhibitor conditions. (A-E) CuS-PEG NPs-induced structure changes of HeLa Cells with and without inhibitor dynasore, CPZ, Filipin and Cyt D in SEIRA spectra by taking cells incubated rGO-Au/Au substrate immersed in PBS as a reference at 2 h. (F) The uptake degree quantified using the ratio of the peak intensity at ~3683 cm$^{-1}$ to ~3255 cm$^{-1}$.

As shown in Figure S6, the peak changes of pretreated with Filipin (D) and Cyt D (G) of CuS-old groups are close to that of the experimental group without any pretreatment (A), indicating that cellular uptake of CuS-old NPs is not caveolae and actin polymerization related phagocytosis and clathrin/caveolin independent endocytosis pathway.\textsuperscript{22, 23} Compare the peak intensities of the two $\nu$(OH) stretching modes at ~3683 and 3255 cm$^{-1}$, we found that the ratio of I$_{3683}$/I$_{3255}$ are in qualitative agreement with the result of CuS-old group in ICP-MS (Figure 4F, the clathrin-mediated endocytosis), suggesting that the uptake degree could be roughly
evaluated using the ratio of at ~3690-3670 cm$^{-1}$ to 3270-3250 cm$^{-1}$. 
Figure S7. (A-B) The optical microscope images for cells before (A) and after (B) illumination on the rGO-Au/Au substrate.

After continuous illumination, cell shrinkage and cell membrane blebbing were observed using top-view microscope on the rGO-Au/Au substrate, indicating that cells are apoptosis and the spectral marker of apoptosis (PS) may be appeared in the MIR region.
Table S1

| IR frequency (cm\(^{-1}\)) | Tentative assignment |
|-----------------------------|----------------------|
| ~3683 | dangling free -OH bonds or water with less hydrogen-bond network\(^{24, 25}\) |
| ~3470 | asymmetrically -OH stretch mode, and directly adjacent to the hydrophilic lipid headgroup\(^{24-26}\) |
| ~3255 | tetrahedrally symmetric H-bonded bulk water\(^{24-26}\), \(\nu(\text{N-H})\) of protein (amide A)\(^{27}\) or other |
| ~1695-1672 | Amide I band of \(\beta\)-turns and antiparallel \(\beta\)-pleated sheets\(^{21, 27, 28}\) |
| ~1663, 1658 | Amide I band of 3\(_{10}\)-helical and \(\alpha\)-helical structure\(^{27, 29}\) |
| ~1647 | Amide I band of random coil\(^{21, 28, 29}\) |
| ~1621 | Amide I band of parallel \(\beta\)-pleated sheets\(^{27-29}\) |
| ~1601 | \(\delta(\text{N-H})\) bending vibration\(^{19-21}\) |
| ~1558 | \(\nu_{\text{asym}}(\text{COO}^-)\)^{19-21} |
| ~1542 | Amide II band\(^{27-29}\) |
| ~1461-1449 | \(\delta(\text{CH}_2)\), \(\nu_{\text{sym}}(\text{COO}^-)\)^{20, 27, 29, 30} |
| ~1391 | \(\delta(\text{CH}_3)\), \(\nu(\text{N-C})\)^{20, 29, 30} |
| ~1358 | \(\omega(\text{CH}_2, \text{CH}_3)\) from phospholipid, fatty acid, triglyceride, amino acid\(^{28, 30}\) |

\(\nu\), stretching; \(\delta\), deformation; \(\rho\), rocking; \(\tau\), twisting; \(\omega\), wagging; sym., symmetrical; asym., asymmetrical; His, histidine

Table S1. Tentative assignments of CuS NPs induced HeLa cell peaks in the mid-IR regime.\(^{20, 21, 24-30}\)
Table S2

| IR frequency (cm$^{-1}$) | Tentative assignment |
|--------------------------|----------------------|
| ~3607-3620               | non-hydrogen-bonded $\nu$(OH)$^{24,25,31}$ |
| ~3390                    | hydrogen-bonded $\nu_{\text{sym}}$(OH)$^{24-26}$ |
| ~3248-3300               | strongly hydrogen-bonded $\nu_{\text{sym}}$(OH)$^{24-26}$ |
| ~2965, 2875              | $\nu_{\text{sym}}$(CH$_3$), $\nu_{\text{asym}}$(CH$_3$)$^{20,27,29}$ |
| ~2940, 2846              | $\nu_{\text{sym}}$(CH$_2$), $\nu_{\text{asym}}$(CH$_2$)$^{20,27,29}$ |
| ~1709                    | $\nu$(C=O) may from PS$^{27,32}$ |
| ~1673, 1663              | Amide I$^{27,29}$ |
| ~1643, 1622, 1587, 1495  | $\nu$(C=O), $\delta$(NH$_2$), $\nu$(C=C) ring vibration of Phe$^{33,34}$ |
| ~1557                    | Amide II$^{27,29}$ |
| ~1432                    | $\delta_{\text{asym}}$(CH$_2$), $\delta_{\text{asym}}$(CH$_3$)$^{20,30}$ |
| ~1398                    | $\nu_{\text{sym}}$(COO$^-$), $\delta_{\text{sym}}$(CH$_3$), $\delta_{\text{sym}}$(CH$_2$)$^{20,21,27}$ |
| ~1358                    | $\omega$(CH$_2$) from phospholipid or amino amide side chain$^{20,29,30}$ |
| ~1287                    | ring $\nu$, $\tau$(CH$_2$,CH$_3$)$^{30,35}$ |

$\nu$, stretching; $\delta$, deformation; $\rho$, rocking; $\tau$, twisting; $\omega$, wagging; sym., symmetrical; asym., asymmetrical; Phe, phenylalanine.

Table S2. Tentative assignments of Hyperthermia-induced HeLa cell peaks in the mid-IR regime.$^{20,25-27,29-35}$
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