Smart Plasmonic Nanorobot for Real-Time Monitoring Cytochrome c Release and Cell Acidification in Apoptosis during Electrostimulation

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Supporting Information

ABSTRACT: Cytochrome c (Cyt c) release and cellular pH change are two important mediators of apoptosis. Effective methods to regulate or monitor such events are highly desired for apoptosis research and cancer cell therapy. Herein, we exploited electrostimulation to regulate cellular Cyt c release and apoptosis process, and by designing and preparing a smart and efficient plasmonic nanorobot (with surface-modified Cyt c-specific aptamer and 4-mercaptobenzoic acid) that is capable of Cyt c capture and self-sensing, we achieved real-time SERS monitoring of dynamic Cyt c release and simultaneous cell acidification in apoptosis during electrostimulation. Distinctly different molecular stress responses in the two events for cancerous MCF-7 and HeLa cells and normal L929 cells were identified and revealed. The method and results are valuable and promising for apoptosis and Cyt c-mediated biology studies.

Apoptosis is the process of programmed cell death that controls cell homeostasis and serious disorders, such as cancer and autoimmune diseases. This death process is complicated and involves a large variety of cellular molecules, so revealing (external or internal) stimulation-induced cell apoptosis pathways associated with specific molecules is vital for cancer theranostics and has been a huge challenge. For instance, Cyt c, which plays an important role in the electron transport chain in mitochondria, releases from mitochondria to the cytosol in apoptosis process. The Cyt c release is a major trigger of cascade activation and has been treated as a highly specific event in apoptotic signaling. However, real-time monitoring Cyt c release during apoptosis processes has remained an important challenge. In addition, the pH of cells was also another significant regulatory mediator, which was decreased (acidification) during the apoptotic process. Therefore, real-time monitoring these two events in living-cells is very important for apoptosis research. On the other hand, artificially controlled cellular release of Cyt c might be a solution to cancer therapy. So far, controlled Cyt c release has been achieved and regulated chemically by external drugs, which usually lacks of precise control and has site-effects. Therefore, it is highly desired to develop an alternative method that capable of controlling Cyt c releasing and regulating cell apoptosis.

In this study, we exploited a physical way to regulate on-demand cellular release of Cyt c and programmed cell death by using electrostimulation (ES), which has been applied for other biomedical applications, such as nerve repair and tissue regeneration. The ES method is supposed to have an advantage over drugs to realize fast on-demand cellular release of Cyt c at desired time and dose (to induce programmed cell death), by controlling the treatment time and voltage of ES. By designing a smart plasmonic nanorobot that capable of Cyt c capture and sensitive Cyt c and pH detections, we have studied dynamic molecular stress responses of the two events in living-cells during the slight ES process (~1 V applied bias), and revealed differential molecular stress responses for cancerous MCF-7 and HeLa cells and normal L929 cells during the ES-induced apoptosis process.

As an emerging sensitive bioassay technique that capable of real-time monitoring dynamic biological processes in cells, surface-enhanced Raman scattering (SERS) has been recently

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exploited for biosensing of cancer biomarkers and intracellular pH distributions. It performs considerable superiority in monitoring the varying subcellular environments and molecules, but it is quite dependent on the nanoprobe design. Therefore, to monitor dynamic Cyt c release and pH changes and unveil apoptosis mechanisms of cells during the ES process, a smart Cyt c and pH dual-responsive SERS nanoprobe was designed and prepared based on Cyt c-specific aptamer and 4-mercaptopentanoic acid (4-MBA) comodified AuNPs. As schematically shown in Scheme 1, SERS detection of Cyt c can be achieved through C–S vibrational modes change of the MBA-AuNP caused by photoinduced electron transfer (ET)13–15 between thiolated AuNPs and specifically captured Cyt c that dynamically released from mitochondria to cytoplasm during the ES process. In our work, we found that besides cell acidification, the amount of Cyt c trigger-released in single cancer cells were found at least 1–2 magnitudes higher than that in single normal L929 cells after the ES process. And direct real-time visualization of MMP depolarization and Cyt c releasing/distribution in apoptotic cells was also achieved during the ES process.

**EXPERIMENTAL SECTION**

**Materials.** Tetrachloroauric acid trihydrate (HAuCl₄·3H₂O), sodium chloride (NaCl) sodium citrate (C₆H₇Na₃O₇), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 4-mercaptoethanol, 4-mercaptobenzoic acid (4-MBA) were purchased from Aladdin. The polypeptide (PLL) and dimethyl sulfoxide (DMSO) were bought from Key-GenBioTech. The methoxypolyethylene glycol thiol (mPEG-SH, MW 5000), poly(dimethylallyl ammonium chloride) (PDDA), Annexin V-FITC apoptosis detection kit and calcein-AM/PI suitable for fluorescence were bought from the Sigma-Aldrich (USA). The 5,5',6,6'-tetrahydro-1,1',3,3'-tetraethylimidacarbocyanine (JC-1) and Hoescht 33424 were bought from the Invitrogen (Carlsbad, CA). The Reactive Oxygen Species (ROS) Assay Kit was purchased from Sigma-Aldrich (USA). The lysosomal extraction kit was bought from BestBio (Shanghai). The Dulbecco’s modified Eagle’s medium (DMEM), antibiotic solution, fetal bovine serum (FBS) and 0.25% trypsin/2.2 mM EDTA solution were purchased from Ding Guo biotech Co., Ltd. The DNA aptamers to Cyt c, which were modified by different methods and the nonaptamer DNA, were synthesized from Sangon Biotech Co. Ltd. (shanghai, China). The two sequences used for aptamer to Cyt c were 5'-Cy3-CCG TGT CTT GGG CCG ACC GGC GCA TGG GGT ACG TTG C-3' and 5'-SH CCG TGT CTT GGG CCG ACC GGC GCA TGG GGT ACG TTG C-3', respectively.18 Moreover, the sequences of nonaptamer was designed to 5'-SH GTC ACA CAT GAC CTC TGA AGT.17

**Instrument.** The gold nanoparticles (AuNPs) were characterized through Hitachi 600 transmission electron microscope (TEM). The UV–vis absorption spectra were taken using a Lambda 750 spectrophotometer (Perkin–Elmer). The concentration of the AuNPs was detected using the ICP-MS (Thermo Scientific Icap 6300). CHI 660E electrochemical workstation was purchased from the Chenguang (Shanghai). The dynamic light scattering (DLS, Zetasizer Nano ZS 90) was purchased from British Ma Erwen Co., Ltd. An inverted microscope (Leica DMI6000B, Germany) has an external double channel optical system. Dark-field images were conducted through this microscope with a dark-field condenser (Leica 0.90 S1). The fluorescence module has three excitation wavelength ranges that were A4 (360 ± 40), LS (480 ± 40), and N3 (546 ± 12), respectively. All the pictures are obtained using the Leica DFC450 C digital camera. The pH measurements were performed through a pH-FE28 digital pH-meter (Mettler-Toledo Instruments (shanghai) Co., Ltd.) with a combined glass-calomel electrode. The ESCALAB-MKII spectrometer (VG Co., UK) with Al KR X-ray radiation as the X-ray source for excitation was used for conducting the X-ray photoelectron spectroscopy (XPS). FT-IR spectrum was performed using a Bruker Vertex 80 V spectrometer.

**Preparation of Aptamer/MA-AuNPs SERS Nanorobots.** First, the MBA (1.0 × 10⁻⁴ M, 100 μL) was assembled on the surface of AuNPs (0.1 nM, 10 mL) through the Au–S under the vigorous stirring for 2 h, after which the solution was centrifuged at the 8500 rpm for 10 min by twice and then resuspended into the deionized water under the same concentration. The aptamer to Cyt c (50 μM, 100 μL) solution was added to the MBA-AuNPs prepared solution (10 mL) to stir for 4 h at room temperature. Then the solution was centrifuged at 8500 rpm for 10 min by twice and stored at 4 °C for future use. The coverage of MBA on AuNPs was determined by SERS based on the peak intensity of MBA at 1586 cm⁻¹ with reference to a standard MBA solution in ultrapure water. The concentrations of standard MBA solution were detected from the 1.0 × 10⁻⁸ to 1 × 10⁻⁴ M (Figure S3a). Ten spectra of the mean SERS intensity at 1586 cm⁻¹ of different concentrations were randomly selected for fitting (Figure S3b). At the same time, the content of the DNA aptamer to Cyt c was measured before and after the reaction using the microplate reader (Figure S4). The concentration of MBA absorbed on the AuNPs (0.1 nM) was estimated to be ~1.74 × 10⁻³ M. According to the surface area formula of a sphere (4πr³/3) for AuNPs with diameter of ~20 nm, the superficial area of a single AuNP is calculated to be ~1.3 × 10⁻¹⁵ m². Assuming that the particles are monodisperse, the total surface of AuNPs (0.1 nM, 10 mL) was calculated to be ~7.83 × 10⁻⁴ cm². The number of MBA covered on the AuNPs was therefore calculated to be ~1.05 × 10⁻⁸, with the coverage of MBA on AuNPs surface estimated to be ~1.34 × 10⁻¹⁵ cm⁻². On the basis of the average area of a single AuNP, the number of MBA molecules on a single AuNP was estimated to be ~1.74 × 10⁵ particle⁻¹. Likewise, the number of aptamer molecules on a single AuNP was estimated to be ~828 particle⁻¹.

**Characterization of Cyt c Captured by Nanorobots Using Infrared Spectra.** To check Cyt c capture ability of the as-prepared aptamer/MA-AuNPs SERS nanorobots, the 10 μL of Cyt c (1.5 mM) solution was added into the 990 μL of SERS nanorobots to mix for 30 min at the room temperature. Then, the solution was centrifugated at 8500 rpm for 10 min by twice to get purified Au-nanoprobes, which was redispersed into deionized water. Lastly, the colloidal solution was dripped on calcium fluoride tablets to detect from...
900 to 4000 cm$^{-1}$. The blank calcium fluoride tablet was set as background sample.

**Sensing and Selectivity of Nanorobots.** The solution of Cyt c was freshly prepared, and 10 μL of Cyt c solution at different concentrations was immediately added into 90 μL of the as-prepared aptamer/MBA-AuNPs (0.1 nM) in 10× PBS (10 mM, pH 7.4) followed by incubation for 30 min at 37 °C. The resulting mixture was detected using the confocal Raman spectrometer. The laser power was 7.1 mW while the integration time was set for 10 s and the integral number was once under the excitation wavelength of 633 nm. All the SERS experiments were performed at the same test condition under ambient conditions. The selectivity of nanorobot was conducted by measuring the SERS responses of possible interferents in the absence or presence of Cyt c. The L-glucose (10 mM), VC (0.1 mM), Aβ 40 (1 μM), GSH (0.1 mM), trypsin (0.1 mM), biotin (0.1 mM), myoglobin (0.1 mM), and Cyt c (0.1 μM) were performed to investigate the selectivity of the nanoparticles for Cyt c.

**Cell Culture.** HeLa cells (cervical cancer), MCF-7 cells (breast cancer), and L929 cells (mouse lung fibroblasts) were bought from the American Type Culture Collection (ATCC, USA). HeLa, MCF-7 and L929 cells were grown in the Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO$_2$.

**Electrostimulation for Different Cell Lines.** Electrostimulation of cells was performed in a standard three electrode system on a CHI 660E electrochemical workstation at room temperature. The Ag/AgCl (KCl saturated) and Pt were selected as the reference electrode and counter electrode, respectively. The NaCl (0.9 wt.%), which can be formed to isosmotic pressure with cells was mixed with complete medium. The NaCl (0.9 wt.%) was added into a 1 mL buffer solution to incubate the cells for 20 min at the 37 °C. After that, the rest of the nanoparticles in medium were removed and the cells were washed using the PBS solution by three times. The cells with different constant voltages (0.3, 0.6, 0.9, and 1.2 V) for 5 min under the constant voltage stimulation. After the process, the MCF-7 cells seeded on the ITO glasses were cultured for 12 h and was dried by N$_2$. ITO electrode modified by the polylysine (PLL, 1 wt.%) for 5 min at the room temperature, which was washed using the deionized water and was dried by N$_2$. ITO electrode modified was disinfected using the ultraviolet radiation for 30 min to capture the cells because the surfaces of cells are negatively charged. The cells (5.0 × 10$^4$) on the ITO glass were cultured for 12 h at 37 °C in a humidified atmosphere containing 5% CO$_2$. After that, the cells were stimulated to apoptosis under the constant voltage.

**Mitochondrial Membrane Potential of MCF-7 Cells during ES Process.** To investigate the changes of mitochondrial membrane potential (MMP) during the ES process, the MCF-7 cells seeded on the ITO glasses were treated with different voltages (0, 0.3, 0.6, 0.9, and 1.2 V) for 5 min under the constant voltage stimulation. After the stimulation, the cells were washed using the PBS solution (10 mM, pH = 7.4) by three times, then the cells were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocya-nine iodide (JC-1) which is an ideal fluorescent probe widely used to detect mitochondrial membrane potential. A 10 μL of JC-1 (200 μg/mL) solution was added into a 1 mL buffer solution to incubate the cells for 20 min at the 37 °C. After that, the cells were washed using the PBS solution by three times and observed with 40× objective through a fluorescence microscope.

**Monitoring of Cyt c Releasing and pH Reducing within Cells by Nanorobots under the ES Process.** Briefly, the different cell lines (5.0 × 10$^4$ cells) were seeded on the ITO glasses to culture overnight. The aptamer-Cyt c@MBA-AuNPs (0.1 nM) probes were redispersed into the 1 mL complete medium to incubate with different cell lines (MCF-7, HeLa, and L929 cells) for 6 h. After that, the rest of the nanorobots in medium were removed and the cells were washed using the PBS solution by three times. Finally, the cells were treated with the probes were stimulated under the constant voltage and the sensing of Cyt c and pH was traced using SERS technique in this process with different voltages (0.3, 0.6, 0.9, and 1.2 V) for 5 min or with varied duration (0, 1, 3, and 5 min) under the 0.9 V stimulation. The SERS spectra were collected during the NIR laser exposure period by a confocal Raman system (LabRAM ARAMIS, HORIBA JobinYvon, USA) with a 7.1 mW/633 nm laser. The spot size was 1.5–3 μm and outstretched scan spectra with a spectral from 400 to 1800 cm$^{-1}$ were recorded with an integration time of 10 s and one accumulation.

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**RESULTS AND DISCUSSION**

**Electrostimulation for Different Cell Lines to Apoptosis.** We applied a constant potential field to living-cells cultured in an electrochemical cell to stimulate cell apoptosis. Three different cell lines (cancerous MCF-7, HeLa, and normal L929 cells) were randomly selected and stained with live/dead fluorescence dyes (calcium-AM/propidium iodide (PI)) to check cell viability, and the cell viability was also tested by MTT assay. As shown in Figure 1, the cells were gradually died with the voltage increasing from 0.3 to 1.2 V for 5 min duration, but it is more conducive to the apoptosis of cancer cells than normal cells. The method is, therefore, promising for potential cancer therapy applications. In the following study, to reduce damage to normal cells and for potential cancer therapy applications, optimized time of ES stimulus at constant 0.9 V for 5 min (Figure S1) was selected to trigger cell apoptosis.
Characterization of Nanorobots Prepared. To monitor Cyt c release and pH changes and unveil apoptosis mechanisms of cells during the ES process, a smart plasmonic nanorobot that Cyt c/pH dual-detection was designed and prepared based on Cyt c-specific aptamer and 4-mercaptobenzoic acid (4-MBA) co-modified AuNPs, as schematically shown in Scheme 1. To our knowledge, dual-responsive SERS nanosensor for real-time monitoring Cyt c release and pH change in living-cells during ES process has not been reported. The successful construction of the Au-nanoprobe (∼20 nm) acted as smart nanorobot was characterized by UV−vis spectra and zeta potential changing (Figure 2a and b). Moreover, the (low) cytotoxicity of the nanorobots was also assessed by MTT assay (Figure 2c) and the morphology of nanorobots was spherical, as seen from Figure 2d. The number of MBA (whose SERS bands were assigned in Figure S2 and Table S1) and Cyt c-specific DNA aptamer assembled on a single AuNP were estimated to be ∼1.74 × 10^5/AuNP and ∼828/AuNP, by SERS analysis and microplate reader, respectively (Figure S3 and S4).

Calculating the Energy Levels of the AuNPs/MBA/Cyt c (Fe³⁺) Nanosystem. In the AuNPs/MBA system, the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) levels of MBA was reported to be 6.24 and 1.68 eV, while the Fermi level of AuNPs was 4.24 eV. In this case, the energy barrier from the Fermi level of Au to LUMO of MBA is 3.18 eV that is more than the incident photon energy (1.96 eV), so that no electron transfer between Au and MBA occurs. While in the Au/MBA/Cyt c (Fe³⁺) system, the conduction band (CB) and the valence band (VB) level of (Cyt c) Fe³⁺ are at 4.19 and 6.52 eV respectively. In this case, the energy barrier from the Fermi level of Au to CB of (Cyt c) Fe³⁺ is 0.05 eV and incident photon (1.96 eV) has enough energy to transfer the excited electrons from AuNPs to the CB of (Cyt c) Fe³⁺, so that the ET pathway was turned on in AuNPs/MBA/Cyt c system. By calculating the energy levels of the AuNPs/MBA/Cyt c (Fe³⁺) nanosystem (Figure 3), ET from AuNPs to conduction band (CB) of (Cyt c) Fe³⁺ occurs as the process is energy favorable.

Characterization of Cyt C Capture Capability of Nanorobots. The smart nanorobots capture surrounding Cyt c, which was confirmed by ICP-MS detection, IR spectra and XPS. As shown in Figure 4a, the (Fe, S) elemental content of the nanoprobes increased obviously after the capturing process; meanwhile the specific bands of Cyt c at 3283, 2954 (−NH₂), 1654 (amide (I)) and 1583 cm⁻¹ (amide II) in Figure 4b were also emerged after the Cyt c captured by nanorobots. Moreover, the Cyt c capture ability of the nanorobot was further confirmed by XPS, as shown in Figure 4c−f. From C 1s spectra, the binding energy at 285.4 eV could be assigned to CH₂−C=O, which was only originated from Cyt c (Figure 4f), and all the bases have no this structure in their molecular formula. The binding energy at 707 eV was entirely attributed to Cyt c other than nanorobots (Figure 4d). A much higher abundance of the binding energy at 165 and 399 eV assigning to S 2p and N 1s was caused by Cyt c captured by nanorobots (Figure 4e and f). The capture
348 capability of nanorobots to Cyt c was also approved using
349 UV−vis spectra at 408 nm, which was the absorption band of
350 Cyt c (Figure S5a). The capturing rate for Cyt c of the
351 nanorobot was also estimated to be ∼91.6% by ICP-MS
352 (Figure S5b). All the conclusions affluently testified that the
353 nanorobots designed owns good capability to capture Cyt c.

**Nanorobots for Cyt c and pH Sensing.** In the ES
355 process of nanorobots, we found that the SERS signal of MBA-
356 AuNPs itself was not interfered (for Cyt C/pH sensing) under
357 0.9 V ES treatment as SERS spectra of the MBA-AuNPs
358 remain the same after the ES process (Figure S6). To further
359 check usability of the nanorobots for Cyt c concentration
360 sensing, Cyt c with varied concentrations from 1 nM to 10 μM
361 (Figure S7a) were tested in vitro. The SERS peak at original
362 1078 cm\(^{-1}\), which was attributed to the C−S stretching
363 vibration of MBA was observed blue-shift gradually to 1071.5
364 cm\(^{-1}\) with the increase of Cyt c concentration, as a result of
365 coordination of MBA sulfur with Cyt c iron which facilitates
366 ET between AuNPs and Fe\(^{3+}\) and leads to electronic structure
367 change of the MBA molecule, and hence, change in the
368 vibration mode of C−S bond. Figure 5a show corresponding
369 SERS spectra (obtained by amplification from the original
370 spectra in Figure S7a) and the fitted linear relationship
371 between concentration of Cyt c and SERS peak shift with
372 respect to 1078 cm\(^{-1}\) (Figure Sb). It is worth noting that
373 although the stability of the instrument was quite good (cf.,
374 Figure S9), since the resolution of Raman instrument is about
375 1 cm\(^{-1}\), the concentration of Cyt c below ∼10 nM is
376 undetectable. The nanorobots also exhibited superior
377 selectivity for Cyt c sensing (Figure S8 a). Moreover, the
378 nanorobot showed a good reliability for pH sensing. As shown
379 in Figure 5c, the SERS intensity ratio \( I_{1572}/I_{1586} \) at 1572 and
380 1586 cm\(^{-1}\) for pH sensing.

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**Figure 4.** (a) Contents of ferrum and sulfur elements contained in nanorobots before and after capturing Cyt c (150 μM) for 30 min. (b) The IR spectra of Cyt c solution (1), 4-MBA solution (2), and the purified SERS-responsive nanorobot before (3) and after (4) capturing Cyt c. (c, d) The XPS spectra of C 1s, Fe 2p of nanorobots after capturing Cyt c (10 μM). (e, f) XPS spectra of S 2p, N 1s of nanorobots before and after capturing Cyt c (10 μM).

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**Figure 5.** (a, b) Corresponding nanoprobe SERS spectra evolution (peak shift at 1078 cm\(^{-1}\)) and the fitted linear relationship for Cyt c concentration sensing. The SERS peak at original 1078 cm\(^{-1}\) and after the blue-shift was denoted as \( S_0 \) and \( S \), respectively. (c, d) The corresponding nanoprobe SERS spectra evolution and the linear relationship with respect to SERS intensity ratio \( I_{1572}/I_{1586} \) at 1572 and 1586 cm\(^{-1}\) for pH sensing.

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as shown in Figure S7a and b, both the SERS band at 1078 cm\(^{-1}\) for pH measurement and the SERS band at 1586 cm\(^{-1}\) for Cyt c sensing had no detectable changes. This renders the as-prepared nanorobots reliable for dual monitoring of Cyt c release and pH changes in cells during the ES-induced apoptosis process.

**Real-Time Visualization of MMP and Cyt c Releasing/Distribution under ES with Different Voltages.** Cyt c is known one of the important small heme-containing metalloprotein existing in the cytosol between the inner and outer membranes of mitochondria. Upon decreasing mitochondrial membrane potential (MMP) of cells under ES, the Cyt c will release from mitochondria to cytoplasm and last it enters into...
the cell nucleus to induce cell apoptosis.\textsuperscript{4,8} Consequently, we first monitored simultaneously in real-time the mitochondrial membrane potential (MMP) depolarization (cf., Figure S10) and Cyt c release/distribution in apoptotic MCF-7 cells (cf., Figure S11). The former was achieved by introducing fluorescent JC-1 dye, while the latter was monitored with a fluorescence turn-on tactic (Figure 6a) by using another Cyt c nanoprobe based on AuNP and Cyt c-specific aptamer (differing with the SERS Au-nanoprobe (nanorobot) in Scheme 1). Figure 6b and c show the corresponding JC-1 fluorescence imaging of the MMP and fluorescence imaging of the Cyt c release/distribution activated in MCF-7 cells under different ES voltages for 5 min. As seen from Figure 6b, the red fluorescence of J-aggregated dyes in the mitochondrial membrane gradually reduced and finally disappeared, while green fluorescence of JC-1 monomer was gradually increased with the voltage increasing from 0 to 1.2 V, indicating the loss of MMP during the ES process. Figure 6c shows dynamic release and real-time cellular distribution of Cyt c during the ES process. With the voltage increasing from \( \sim 0.6 \) to 1.2 V, pronouncedly more Cyt c were triggered to release into the cytosol and turn-on red fluorescence of the Cyt c nanoprobes. We also monitored in real-time the dynamic expression of Cyt c and pH under a constant voltage of 0.9 V (Figure S11b). Figure S12 shows the corresponding flow cytometry data analysis. Since oxidative stress is a negative effect of free radicals produced in cells and tightly regulated by Cyt c phosphorylation,\textsuperscript{23} we also examined the possible cellular oxidative stress response during ES. The oxidative stress of cells under the 5 min ES process was found having negligible influences on Cyt c sensing of nanoprobes and cell death as the reactive oxygen species (ROS) within MCF-7 cells was not detected under 0.9 V ES treatments for 5 min (Figure S13).

Monitoring Cyt c Release and pH Changes with Nanorobots in Real-Time during the ES Process. We then applied the nanorobots to track and monitor Cyt c release and pH change in real-time during the ES process. First, the quantity of probes devoured by MCF-7 cells was optimized for 6 h (Figure S14). Most of the internalized nanorobots were found located in the cytoplasm region by bio-TEM (Figure S15). The percentage of the nanorobots actually in cytosol was further calculated by getting rid of the endocytosis pathway of lysosomes. The contents of nanorobots swallowed with lysosomes extracted from MCF-7 cells was measured by ICP-MS, with the percentage of nanoprobes in cytosol estimated to be \( \sim 95.8\% \), as seen in Figure S14c. The time evolution of SERS spectra of the three tested cell lines under different ES potentials (Figure 7a) and Figure 7b and c shows the corresponding SERS responses for the three tested cell lines under different ES potentials (Figure 7a) and Figure 7b and c shows the corresponding SERS responses for the three tested cell lines (L929, MCF-7, and HeLa cells) stimulated with the different voltages. (a1–c1) represent the SERS spectra of the 4-MBA with Cyt c released from cells under ES with different voltage for 5 min. (a2–c2) show the corresponding local amplification regions of the SERS spectra. (b, c) Peak displacement variation at 1078 cm\(^{-1}\) and 1586 cm\(^{-1}\) for Cyt c content sensing and the peak intensity percentages at 1572/1536 cm\(^{-1}\) for pH detection, respectively, of the three tested cell lines under different ES potentials.

The viability of cells after the ES stimulus was estimated from SERS detections according to the obtained linear relationships in Figure 8. The amount of Cyt c trigger-released and pH value of cells before and after the ES process, were estimated from SERS detections according to the obtained linear relationships in Figure 5. The viability of cells after the ES stimulus was compared using the live/dead staining (Figure 8a). Figure 8b shows the corresponding SERS responses for the three tested cell lines. The amount of Cyt c trigger-released in single cancer-cells was at least 1–2 magnitudes higher than that in single normal L929 cells after the ES process, less than 10 nM for L929 cells and \( \sim 1.4 \) and 0.3 \( \mu \)M for MCF-7 and HeLa cells.

Figure 6. (a) Schematic preparation and sensing mechanism of the Cyt c nanoprobes. (b) MMP images of MCF-7 cells stimulated with different voltages for 5 min. The scale bar: 20 \( \mu \)m. (c) The fluorescence imaging of MCF-7 cells after incubating with Cyt c nanoprobes (0.1 nM) for 4 h, under the ES with different voltages for 5 min. The nucleus was dyed with Hoechst3342 (1 \( \mu \)M). Scale bar = 10 \( \mu \)m.

Figure 7. (a) SERS nanorobot for the three cell lines (L929, MCF-7, and HeLa cells) stimulated with the different voltages. (a1–c1) represent the SERS spectra of the 4-MBA with Cyt c released from cells under ES with different voltage for 5 min. (a2–c2) show the corresponding local amplification regions of the SERS spectra. (b, c) Peak displacement variation at 1078 cm\(^{-1}\) and 1586 cm\(^{-1}\) for Cyt c content sensing and the peak intensity percentages at 1572/1536 cm\(^{-1}\) for pH detection, respectively, of the three tested cell lines under different ES potentials.
Figure 8. (a) Live/dead staining of cells stimulated for 5 min under 0.9 V. Scale bar = 50 μm. (b) The SERS spectra of nanosensor treated with the three cell lines before and after ES at 0.9 V for 5 min. (c) The peak intensity percentages of I_{1572}/I_{1586} for pH detection and peak displacement variation at 1078 cm\(^{-1}\) for Cyt c concentration sensing with the three cells before and after the ES.

■ CONCLUSION

We exploited a physical ES method to regulate on-demand cellular release of Cyt c and programmed cell death. By designing a Cyt c/pH dual-responsive plasmonic nanorobot, which has superior selectivity and sensitivity for Cyt c, we studied molecular stress responses of cells during the ES process by monitoring in real-time the cellular pH change and Cyt c release in single apoptotic cells and revealed distinctly different molecular stress responses for cancerous MCF-7 and HeLa cells and normal L929 cells. Besides cell acidification, the amount of Cyt c trigger-released in single cancer cells were found at least 1–2 magnitudes higher than that in single normal L929 cells after the ES process. Direct real-time visualization of MMP depolarization and Cyt c releasing/distribution in apoptotic cells during the ES process was also achieved. The as-prepared nanorobot enables rapid, selective, and sensitive detections of Cyt c and pH changes in apoptotic cells. The method and results are valuable for further unveiling pathophysiological processes and the nature of cancer on cell and molecular levels and provide an invaluable platform for apoptosis and Cyt c-mediated biology studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04027.

Time optimization for cells apoptosis by ES, the SERS spectrum of MBA and assignment, the contents of MBA and aptamer to Cyt c absorbed on AuNPs estimated, the UV–vis spectra of smart plasmonic nanorobot before and after capturing Cyt c and capture rate investigated, the SEM images and SERS spectra of MBA-Au before and after ES, the selectivity of nanorobot, the stability of Raman instrument, the mitochondrial membrane potential images of MCF-7 cells in different time under ES, the flow cytometry analysis of MCF-7 cells before and after ES treatment, the fluorescence imaging of ROS within MCF-7 cells treated with different voltages, the nanorobot uptake by MCF-7 cells with different time and the contents distributed into cytoplasm and lysosomes, Bio-TEM images of MCF-7 cells incubated with nanorobots, the SERS monitoring of Cyt c and pH changes in living cells with different time under the ES (0.9 V), and the repeatability of nanorobots in response to different cells. (PDF)

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■ REFERENCES

(14) Li, M.; Zhang, J. M.; Suri, S.; Sooter, L. J.; Ma, D. L.; Wu, N. Q. 
(17) Manickam, P.; Kaushik, A.; Karunakaran, C.; Bhansali, S. 
(19) Zhang, X. L.; Liu, Y.; Li, P.; Zhao, B.; Cui, B.; Soltani, M. J. 
(21) Dong, A. C.; Huang, P.; Caughey, W. S. Biochemistry 1992, 31, 
182–189.
(22) Moss, D. E.; Breton, J. N.; Mantele, W.; Nabedyk, E. Eur. J. 
(23) Yu, Z.; Chen, L.; Park, Y.; Cong, Q.; Han, X. X.; Zhao, B.; Jung, 
Elena-Real, C. A.; González-Arzola, K.; García-Mauriño, S. M.; De la 
7955–7960.
(26) Qi, G. H.; Wang, Y.; Zhang, B. Y.; Sun, D.; Fu, C. C.; Xu, W. 
(27) Fu, C. C.; Xu, W. Q.; Wang, H. L.; Ding, H.; Liang, L. J.; Cong, 