Quantitative and Real-time Effects of Carbon Quantum Dots on a Single Living HeLa Cell Membrane Permeability

(Supporting information)

Reagents and Materials. All the chemicals were used as received from commercial sources. NaCl, KCl, Na_2HPO_4, KH_2PO_4 and the redox mediator, $K_4[Fe(CN)_6]\cdot 3H_2O$, were all analytical purity. All solutions were made with Milli-Q water (Millipore).

Synthesis of CQDs–OH. The CQDs–OH was obtained by electrolyzing graphite rod in ultrapure water. In this experiment, two graphite rods were vertically inserted into the ultrapure water and the graphite rods were placed parallel with a separation of approximate 7.5 cm. One of the graphite rods is anode and the other is cathode. The potential with 30 V was applied by using a direct current (DC) power supply. Then electrolyzed graphite rod for 5 days with continuous stirring and a dark-yellow solution was obtained. Following, the solution was filtered with slow-speed quantitative filter paper and then the filtered liquor was high-speed (22,000 rpm) centrifuged for 30 min. After that, the solution was dried in 60 ℃ then the CQDs-OH was obtained.

Synthesis of CQDs–PEG. The CQDs–PEG was synthesized by modifying CQDs-OH with 6-arm Polyetheylene Glycol-Amine which is owing to the amount of carboxylic acid functional groups on CQDs-OH surface. The mixed solution was bath-sonicated for 5 min. Then N-(3-Dimethylaminopropyl-N'-ethylcarbodiimide hydrochloride was added to reach 5 mM and the solution continue bath-sonicated for 30 minute. Following, adding enough dichloroethane to reach 20 mM and stirring for 12 hours until Mercaptoethanol was added. The solution was centrifugation in PBS and the supernatant was CQDs–PEG.

Synthesis of CQD–NH_2. CQDs–NH_2 were synthesized by modifying CQDs-OH with ammonia water. The concentration of CQDs-OH was approximately 0.5 mg/ml in our experiment. The volume ratio of CQDs-OH and ammonia water was 1:1 to form a mixed solution which was stirred homogeneously, after which, the target product was hydrothermally synthesized under self-generate pressure. The mixed

solution was sealed in a Teflon-lined stainless steel autoclave and heated at 150 ℃ for 6 h. The obtained light yellow solution was filtered off and placed under fume cupboard for overnight and the CQDs–NH_2 were obtained. Before the experiment, the CQDs–NH_2 were dialyzed until the solution was neutral. Because of the concentration of CQDs–NH_2 is difficult to determine so we use the concentration of the quantum of CQDs-OH instead.

Synthesis of Reduced CQDs–OH. The reduced CQDs–OH was prepared through a simple treatment of the as-obtained water soluble CQDs–OH. A 250 mL flask containing 100 mL of water soluble CQDs–OH solution and 0.1 mL hydrazine hydrate was refluxing with magnetic stirring at 60 ℃ for 4 h. Then the solution was removed to dialysis tube and dialysed for two days. Afterwards, the reduced CQDs–OH were obtained.

Characterization Methods. The transmission electron microscopy (TEM) images of CQDs were characterized with an FEI/Philips Techal 12 BioTWIN TEM. The Fourier Transform Infrared (FTIR) spectrums of CQDs were obtained with a Varian Spectrum GX spectrometer. The Raman spectrum was collected with an HR800 Raman spectroscope (JY, France) which equipped with a CCD detector and laser confocal microscopy. The UV-visible spectra of CQDs were obtained with an Agilent 8453 UV-VIS Diode Array Spectrophotometer.

In order to quantify the functional groups on the surface of the CQDs–OH, we first performed base titration to measure the total quantity of both hydroxyls and carboxylic acid with Hohl's method. In the experiment, 0.05 mol/L NaOH and 0.05 mol/L HCl were used as titrant and results show the total quantity of both hydroxyls and carboxylic acid of CQDs–OH (0.1 mg/mL) was 1.31×10^{-3} mol/L. Then the conductometrictitration (DDS-11Aconductncetitrator) was performed to measure the ratio of these two kinds of functional groups. CQDs (100 mg), 40 mL solvent (Volume ratio: pyridine/acetone = 1/4), 1mL distilled water and 1mL ethanol were pour into a 50 mL 4-mouth flask. The mixture solution was stirred at 150 rpm and by bubbling of air at 25 ℃ for 10 min. Then 0.05 mol/L KOH-isopropyl alcohol standard solution was used as titrant, at the same time automatic signal recorder was opened. Figure S5 show the conductivity-titrantamount curve. And point A and B are the equivalence points of carboxyland hydroxyl, respectively. We get the content of carboxyl and hydroxyl through the calculation formula as follow:

$$n(\text{carboxyl}) = V_A \times c = 1.71 \times 10^{-3}\ \text{L} \times 0.05\ \text{mol L}^{-1} = 8.55e^{-5}\text{mol}$$

$$n(\text{hydroxyl}) = V_B \times c = 25.2 \times 10^{-3}\ \text{L} \times 0.05\ \text{mol L}^{-1} = 1.26e^{-3}\text{mol}$$

Because of the concentration of water soluble CQDs is 0.1 mg/mL, and 100 mg CQDs were titrated, so the content of carboxyl and hydroxyl of water soluble CQDs were 8.55×10^{-5} mol/L and 1.26×10^{-3} mol/L, respectively. Then the ratio of carboxyl and hydroxyl is approximate 1/15.

The quantitative of function group for CQDs–PEG and CQDs–NH_2 was speculated from EDX. The nitrogen element of CQDs–PEG and CQDs–NH_2 both comes from amino. The mass ratio of nitrogen is 2.36% and 10.73% for CQDs–PEG and CQDs-NH_2, respectively. And the concentration of functional group are approximate 1.68×10^{-4} and 7.66×10^{-4}mol/L for CQDs–PEG and CQDs–NH_2, respectively.

Cell Culture. The HeLa cell were grown in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Beijing) supplemented with 10% fetal bovine serum (FBS,) and 1% mixed solution of penicillin and streptomycin (GNM, Hangzhou). The HeLa cells were cultured in a water jacketed incubator (Thermo, USA) with 5% CO_2 and the temperature maintaining at 37 ℃. An Olympus (CKX41, Japan) inverted microscope was used to observe the cell coverage. The HeLa cells were used for experiment when the cell coverage reached about 50%. When obtained appropriate cells, the cells were taken out of the incubator and then washed three times with PBS (phosphate buffer solution, pH=7.4) and the dead cells were washed off. After that, the cells were incubated with PBS at room temperature. After the electrodes fixed, the PBS was replaced by suitable CQDs solution prepared with 4 mM ferrocyanide PBS.

Electrochemistry and electrodefabrication. The SECM data were obtained with the CHI 920C (Shanghai, China) workstation which was fixed an inverted microscopy and used an inverted microscopy to observe the cell coverage and the states of HeLa cells. In addition, the three-electrode system of SECM was used in our laboratory which the ultromicroelectrode of 25μm diameter platinum wire disk was used as working electrode, a 1mm platinum wire was used as counter electrode and the Ag/AgCl was used as reference electrode. All the potential here were reference to Ag/AgCl.

Fluorescence Based Cell Viability. Two kinds of dyes, calcein-AM and PI, were used in this assay. Calcein-AM is a cell-permeant and non-fluorescent compound that is widely used for determining cell viability. Calcein-AM could across an intact cell membrane and react with the intracellular esterase then the AM group

dropped and producing a highly fluorescent membrane impermeant green fluorophore (excitation: 490 nm, emission: 515 nm). On the contrary, PI can't pass through living Hela cell membrane but can across the damaged membrane and react with intracellular nucleic acid to emit red fluorescence inside dead cells (excitation: 535 nm, emission: 617nm). So its red fluorescent was used as a nuclear or chromosome counterstain; stain for dead cells. Then all these emitted fluorescence cells were easily visible though an inverted microscopy (Nikon) which was a useful method to measure both live and dead cells at the same time. In this experiment, 10 μl calcein-AM and 15 μl PI were dissolved into 5 ml PBS, the concentration of calcein-AM and PI are 2 μmol/L and 4 μmol/L, respectively. When these different concentrations CQDs were added, the toxicity test cell viability has also been measured in real time. Based on the results, the SECM can have enough time to measure the effect of CQDs for the living cell membrane permeability.

Approach curves over a single cell. Approach curves were obtained at 0.5 V in the 4 mM ferrocyanide PBS. The tip was moved near cell with a current rate of 80%. Then the solution was replaced by CQDs solution with 4 mM ferrocyanide and the approach curves can be measured for different time to obtain the cell topographic information.

X-scan over a single cell. The HeLa cells were cultured in about 1 cm spot on a 35 mm Petri dish until the cell coverage was about 50%. The approach curves were measured at three different points outside the cells in 4 mM ferrocyanide with PBS and adjust the tilt of SECM to make sure the stage is level. The current rate was 80%. With the help of inverted microscopy, a single cell can be easily found. The SECM tip was positioned above the single cell about 20 μm with a potential of 0.5 V. The x-scan lines can be measured in a constant height with the increase distance of 0.2 μm. When a single cell was identified, different CQDs solution with 4 mM ferrocyanide PBS was added into the solution to replace the redox solution. And a series of x-scans lines were obtained over this single cell.

i-t curves over a single cell. By approach curves the tip was local above the cell. Then the tip position was adjusted to make the tip hold at 10 μm above a single cell with a constant potential of 5.0 V. And the *i-t* curve near the cell membrane was obtained.

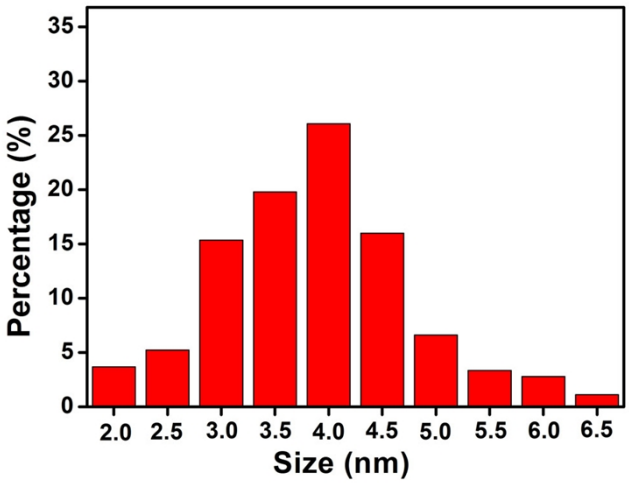

Figure S1. The size distribution of CQDs−OH.

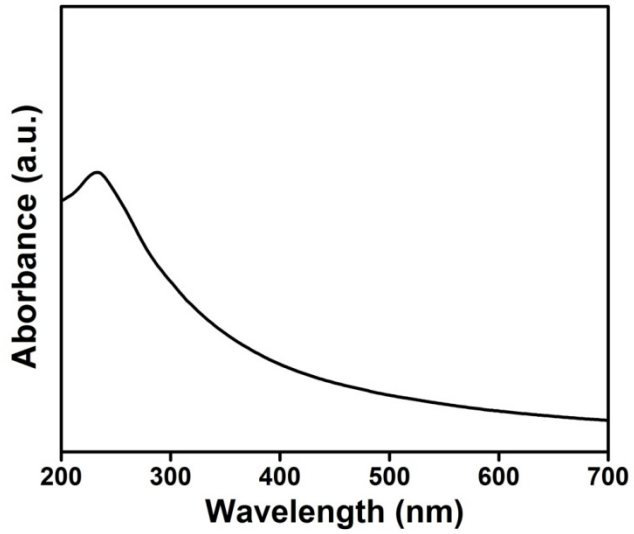

Figure S2. The UV-vis absorption spectrum of 0.1mg/ml CQDs−OH aqueous solution.

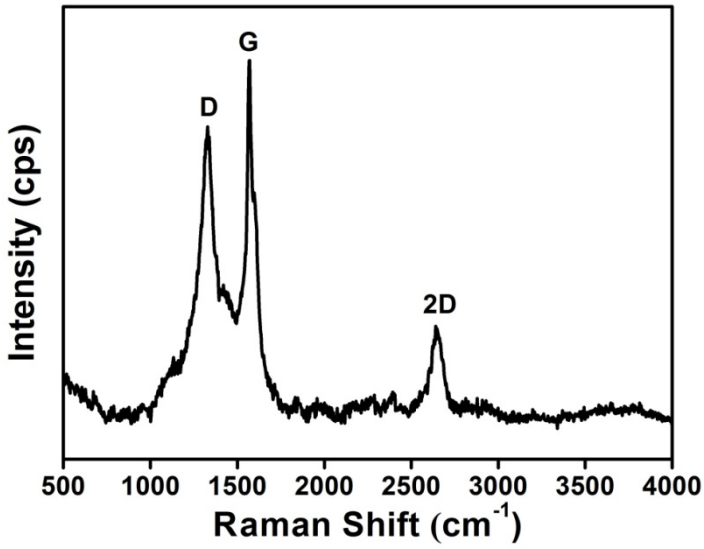

Figure S3. The Raman spectrum of CQDs−OH.

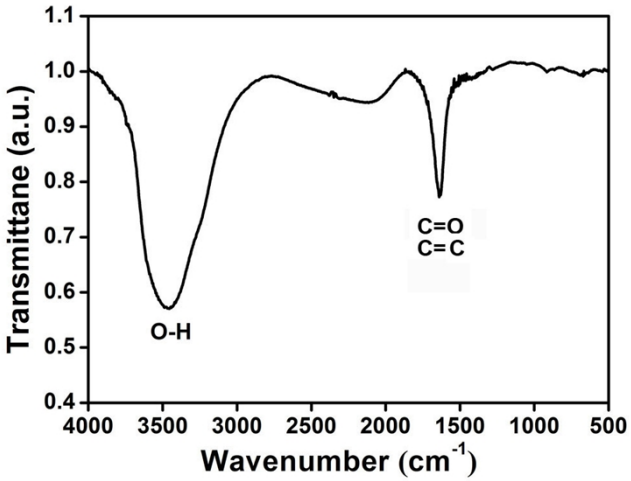

Figure S4. The IR spectrum of CQDs−OH.

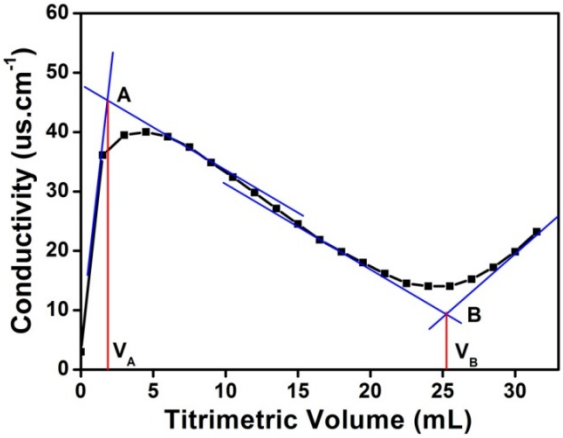

Figure S5. This figure is the conductivity-titrantamountcurve of CQDs−OH. The point A and B are the equivalence points of carboxyl and hydroxyl, respectively.

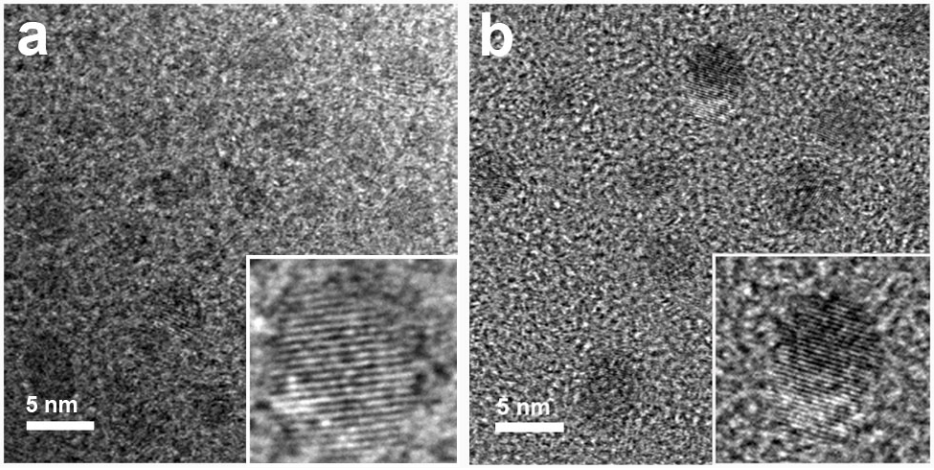

Figure S6. The figure of (a) and (b) are the TEM images of CQDs-PEG and CQDs-NH_2. The inset images are the HRTEM images.

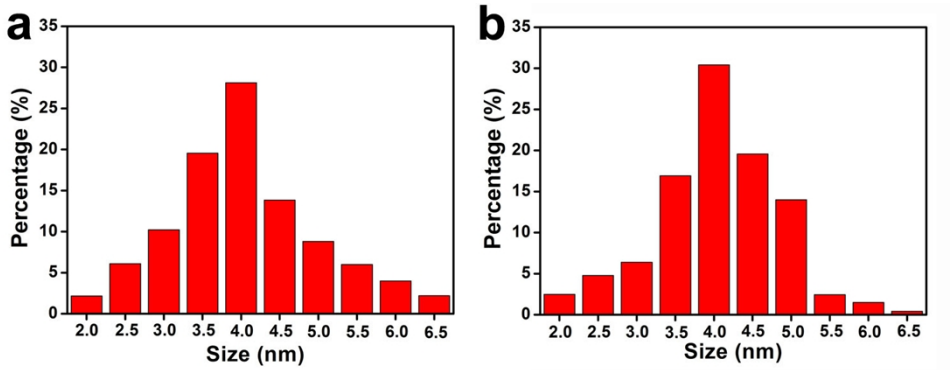

Figure S7. (a) and (b) are the size distribution of CQDs–PEG and CQDs–NH_2, respectively.

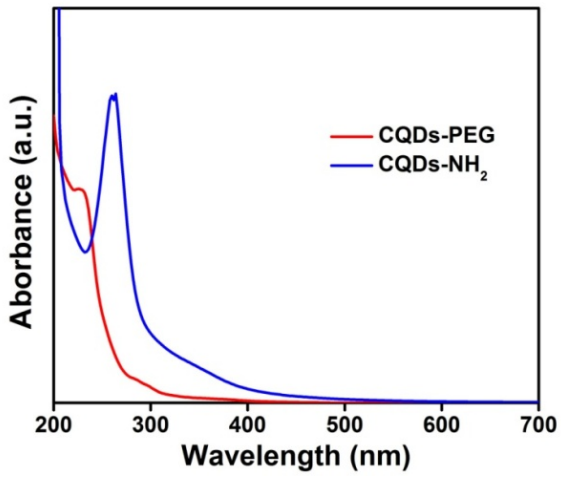

Figure S8. The UV-vis spectra of CQDs–PEG and CQDs–NH_2.

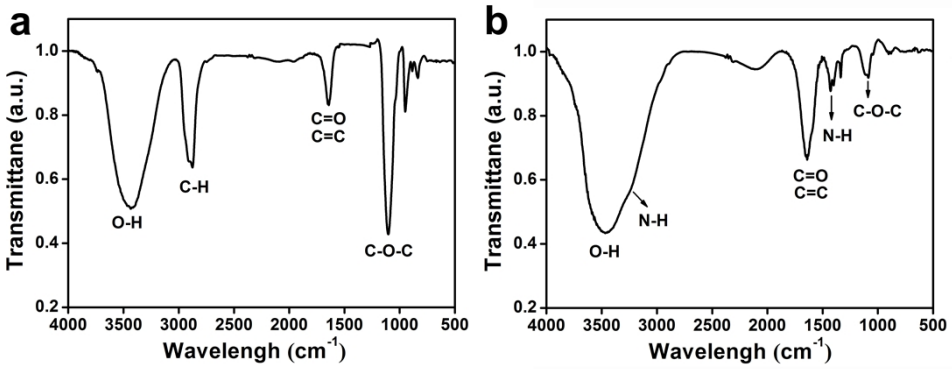

Figure S9. (a) and (b) are the IR spectra of CQDs–PEG and CQDs–NH_2, respectively.

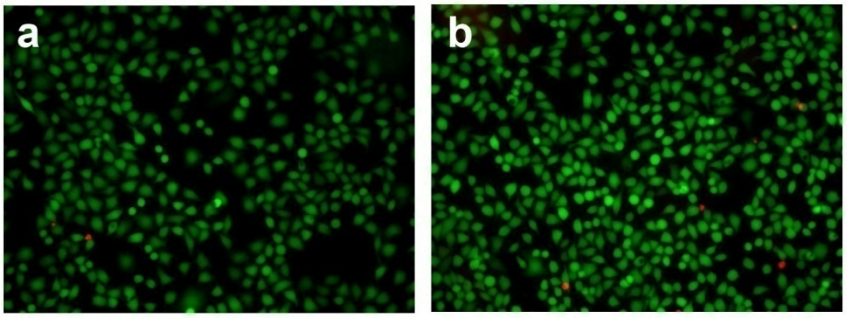

Figure S10. Figure (a) and (b) are fluorescence images of CQDs–PEG, CQDs–NH_2, respectively. The green cells represent living cells and red cells represent dead cells.

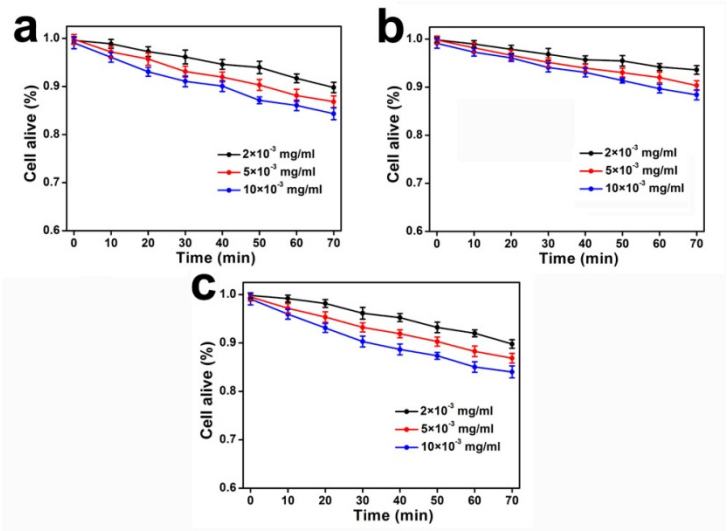

Figure S11. Figure (a), (b) and (c) are fluorescence based viability to HeLa cells for different concentrations of CQDs–OH, CQDs–PEG and CQDs–NH_2 for different period of time, respectively.

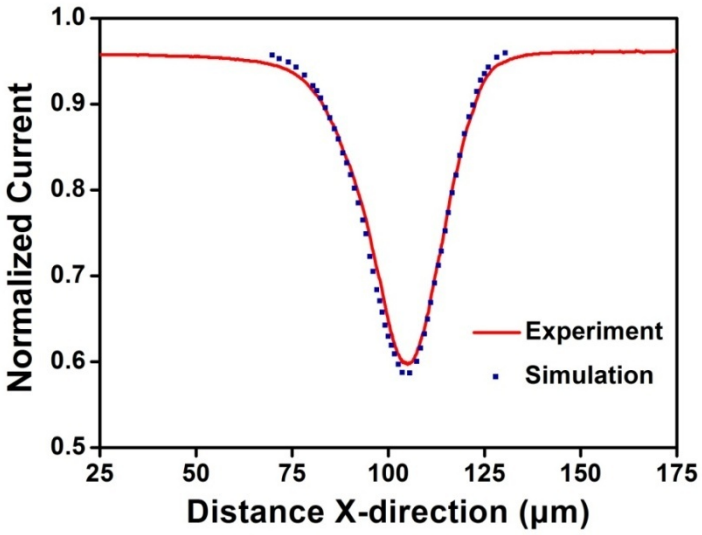

Figure S12. The experiment x-scan line above a single living cell contrast to the simulated x-scan line.

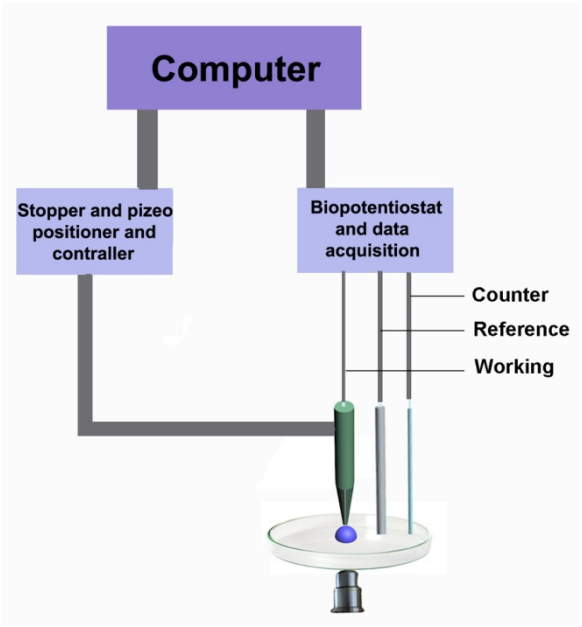

Figure S13. The schematic diagram of scanning electrochemical microscopy for measuring a HeLa cell.

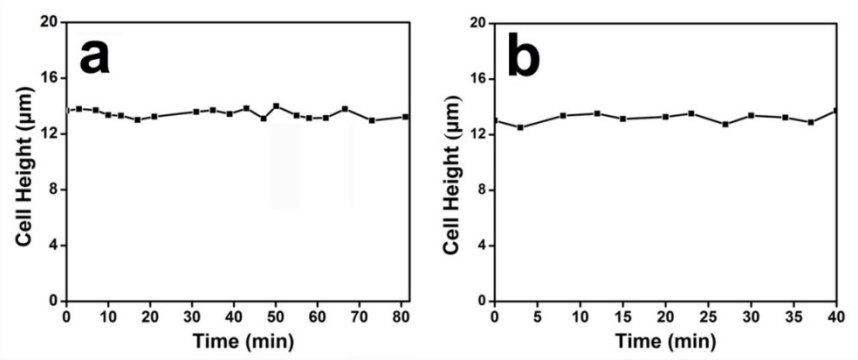

Figure S14. The image of (a) and (b) are the cell height in CQDs–PEG and CQDs–NH_2 solution, respectively.

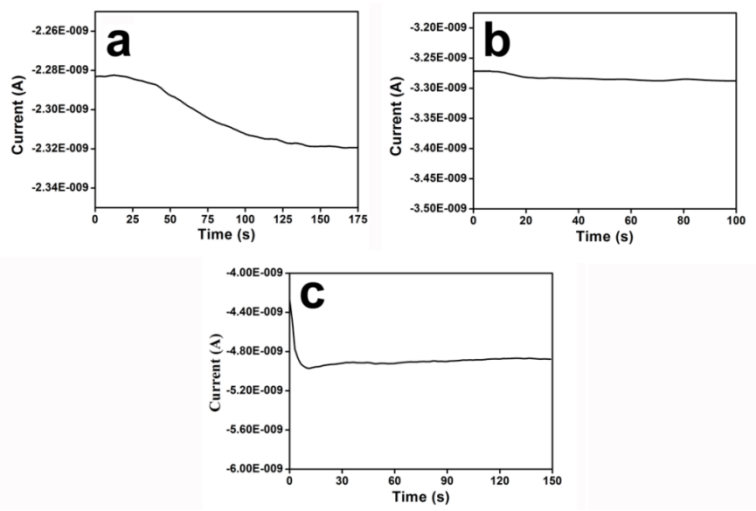

Figure S15. The tip was position above the cell membrane about 20 μm and the current changing was measured. (a) CQDs–OH solution (b) CQDs–PEG solution (c) CQDs–NH_2 solution.

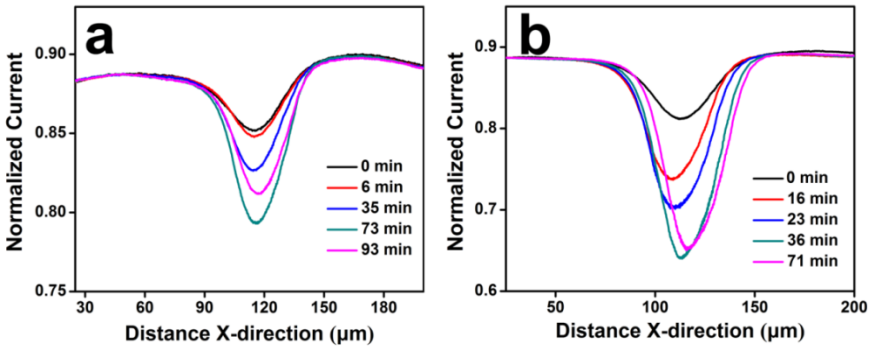

Figure S16. (a) The X-san of a single cell in 2×10^{-3} mg/ml CQDs–PEG solution.(b) The X-san of a single cell in 2×10^{-3} mg/ml CQDs–NH_2 solution.

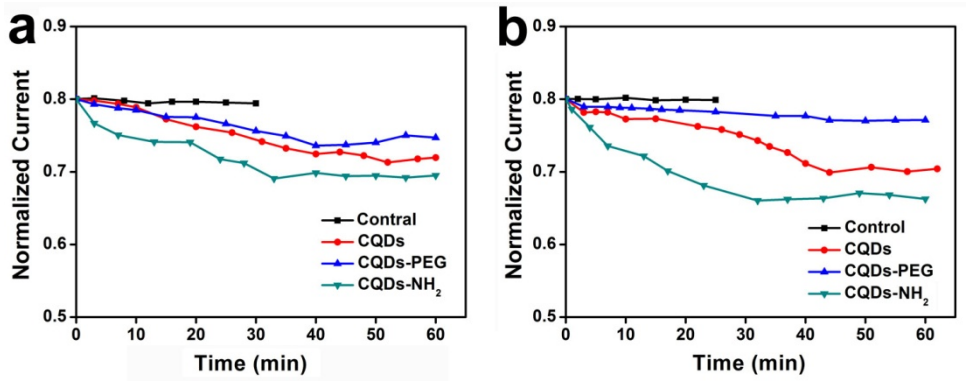

Figure S17. The effect of different concentration of CQDs–OH for Hela cell membrane permeability. (a) 1.5×10^{-3} mg/ml (b)5×10^{-3} mg/ml

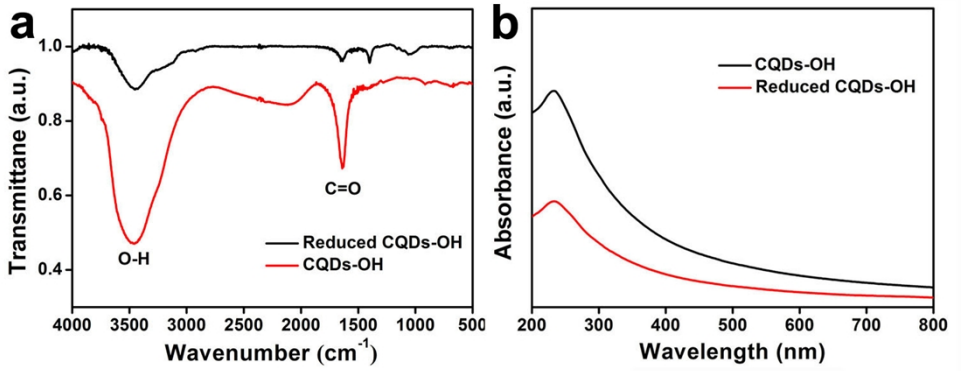

Figure S18. (a) and (b) are the IR spectrum and UV-vies spectrum of CQDs–OH and reduced CQDs–OH, respectively.

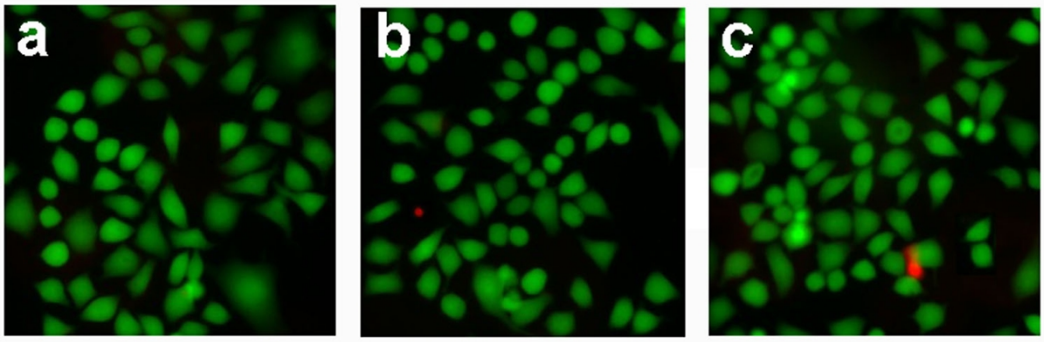

Figure S19. The (a), (b) and (c) are the Fluorescence of HeLa cell in reduced CQDs–OH with a concentration of 1×10^{-3} mg/ml, 2×10^{-3} mg/ml and 5×10^{-3} mg/ml, respectively.

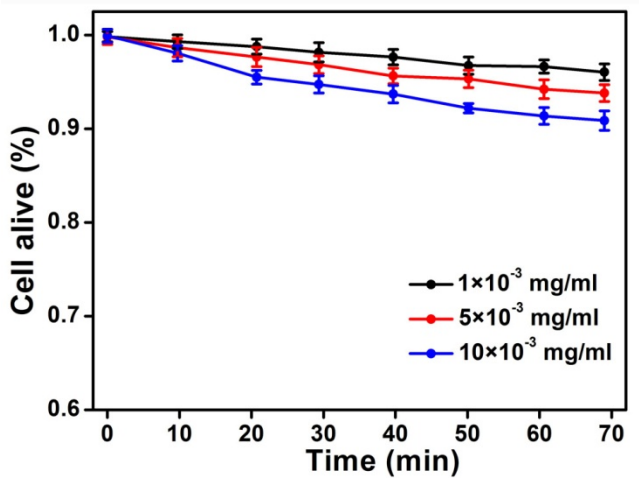

Figure S20. The viability of HeLa cells based upon fluorescence based viability for different concentrations of reduced CQDs for different time.

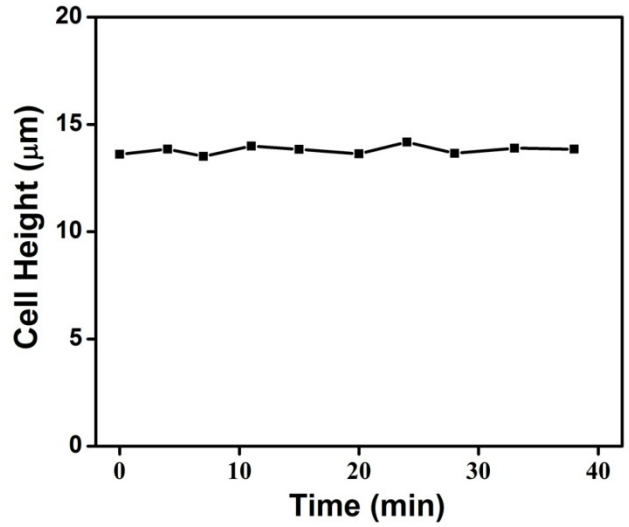

Figure S21. The cell height of a living single cell in reduced CQDs.

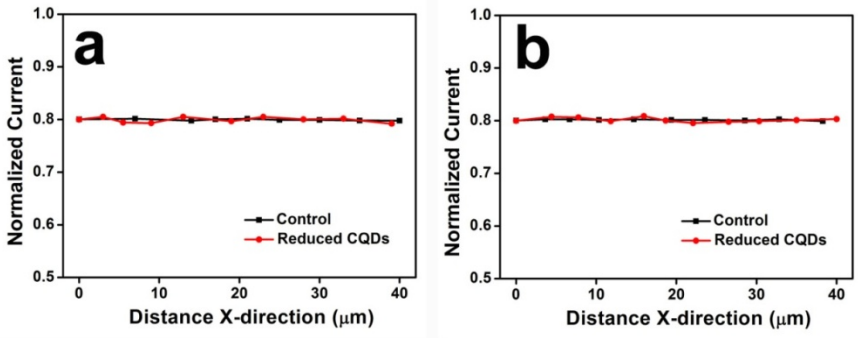

Figure S22. The effect of different concentration of reduced CQDs for Hela cell membrane permeability. (a) 1×10^{-3} mg/ml (b)10×10^{-3} mg/ml

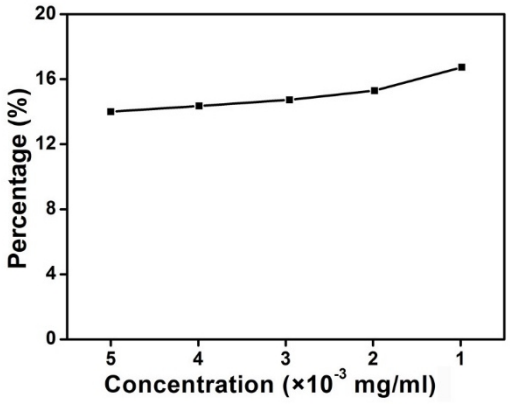

Figure S23. The real-time effect of 5×10^{-3} mg/ml CQDs–NH_2 diluted to 1×10^{-3} mg/ml CQDs–NH_2 with 4 mM ferrocyanide PBS with time. The high concentration was diluted to a lower concentration and detected the current changing. With a series of parallel experiment, the decreasing percentage of minimum normalized current against different concentration was plotted.

Simulation

In the experiment, we assumed that the ferrocyanide present in the solution underwent one electron transfer and the tip was held at diffusion controlled potential to avoid any kinetics complications.

$$R(\text{soln}) - n\text{e} \rightarrow O(\text{soln})$$

Where, $n = 1$, R represents ferrocyanide and O represents ferricyanide.

Because the redox species R and O moved toward and away from the electrode surface only by concentration gradient Fick's second law of diffusion was used in the simulation. The concentration of species R is given as c_R (r,z,t) and the diffusion equation in cylindrical coordinates is described as

$$\frac{\partial c_R}{\partial t} = D\left(\frac{\partial^2 c_R}{\partial r^2} + \frac{1}{r}\frac{\partial c_R}{\partial r} + \frac{\partial^2 c_R}{\partial z^2}\right)$$

Where r and z are the coordinates (Figure S24), t represents time, c and D represents the concentration and the diffusion coefficient of R.

The boundary conditionsat t > 0

At the tip: $0 < r < a$, $z = h1$

$c_R(r, h1) = 0$

$c_O(r, h1) = 4$

At the substrate: $0 < r < r_m$, $z = h2$

$$\frac{\partial c}{\partial z} = 0$$

At the cell membrane: arc1, $d < z < h2$

Flux of R across the acr1 = P (R-$R1$)

P represents the permeability of R across cell membrane in the simulation. R and $R1$ represent the mediator outside and inside the cell, respectively. The glass sheath surrounding the electrode was considered as aninsulator.

At the beginning, the concentration of ferrocyanide inside the cell was zero and the concentration in the bulk solution was 4 mM. Depending on the permeability, the ferrocyanide molecular can across the cell membrane. And the current can be measured with a tip-substrate distance of 20 μm.

$$I_{tip} = \int_{r=0}^{r=a} 2\pi n F D_R r \frac{\partial c_R\,(r,h2)}{\partial z} dr$$

where n=1, F=96485 C/mol and D_R=1 × 10^{-9} m^2/s.

The simulation model described above was solved by finiteelement method where the mesh was increased in exponential grid fashion to generate two-dimensional grid at the regions where sharp change in the concentration gradients were noticed.

X-Scan Simulation. HeLa cell was assumed to be semielliptical shape with symmetry along z-axis as showed in Figure. In this model, permeability was assumed to be zero along cell membrane or arc 1 since topography was the subject of interest here. The 25 μm tip with RG = 10 was also considered symmetrical along z-axis. The tip was held at diffusion controlled potential at all times over the cell or arc 1 and the model was solved in steady state solver condition with the aid of Comsol Multiphysics 4.3 software. The tip to dish distance was maintained at 20 μm at all times. To measure the tip current at different position over the cell, the arc 1 was moved toward left by a distance of 1 μm out of the active simulation sub domain instead of tip moving over the arc 1. This imitates the same condition such as a tip was moving over a single cell in x-direction. Due to symmetry of cell along z-axis, scanning along arc 1 was adequate to obtain the full simulated x-scan over the cell. Both height and radius of cell were considered as adjustable simulation parameters and were fitted with experimental data.

Simulations were done first with P = 0 at arc1 to determine the current at the tip for the certain fixed height of the cell. Then after that different value of P was used in the

simulation to fit the experiment data. The tip current was always calculated with the tip located right above the highest point of the cell height. For example, when P =P_1 the concentration inside and outside the cell was calculated until t =t_1 and then the tip was brought close to the cell top (20μm) away from the dish and held there for 0.1 s (increasing distance 0.2 μm, increasing time 0.02 s) to record the current at t = t_2. The tip was then withdrawn from the top of the cell and the concentration gradient across the cell was again calculated with new value of parameter of P_2.Then the steps were repeated until this experiment finished.In this system, P = KD / L, which P is the velocity of ferrocyanide across cell membrane, K is a constant, D is the diffusion coefficient of ferrocyanide and L is the thickness of cell membrane. Base on the formula, the average value of P was about 6.35×10^{-6} m/s.

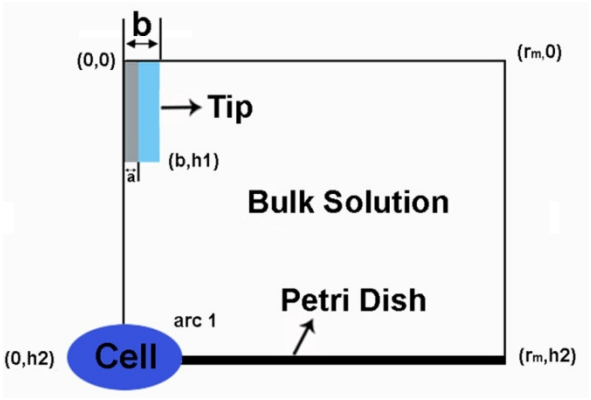

Figure S24. The schematic of simulation model.