

Nano-structure ZnO/Cu₂O photoelectrochemical and self-powered biosensor for esophageal cancer cell detection

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Abstract: The p-n heterojunction photoelectrochemical biosensor, which comprises a p-type Cu₂O film formed by electrochemical deposition and n-type ZnO nanorods formed by the hydrothermal method, is prone to photoelectrochemical reactions and self-powered. Four types of human esophageal cancer cells (ECCs) were detected by this biosensor without requiring an extra bias voltage. The measured photocurrent values of high invasion capacity cancer cells was consistently 2 times higher than those measured by a slight invasion capacity cancer cells. The response time, which was about 0.5 s, allowed repeated measurement.

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OCIS codes: (280.1415) Biological sensing and sensors; (170.1530) Cell analysis; (230.2090) Electro-optical devices.

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1. Introduction

Any common current sensor requires an extra bias voltage. However, the measuring mode may interfere with the actual measurements. To provide an extra bias voltage originally required for detection, a current sensor must be self-powered. The simplest structure for this purpose is a p-n junction photodiode coupled with a simple photoelectric performance analyzer or an electrochemical analyzer for electrical measurement. Compared with common optical measuring instruments, this structure is less expensive and relatively simpler [1]. Most photoelectrochemical (PEC) sensor modules are designed as various composite structures of noble metals (or graphene) and semiconductors or as semiconductor heterostructures [2–4]. Among the available structures, semiconductor heterostructures have the best photocurrent response, which exerts a considerable effect on the transport direction of photo-excited electron-hole pairs, survival distance, recombination rate, etc [5–8]. Thus, the materials, joint structures, and band distributions of the heterostructures may affect their behaviors, especially

in terms of electron–hole separation efficiency and PEC response sensitivity [9]. In 2010, a PEC sensor composed of TiO_2 nanoparticles and FeTPPS on ITO was reported by Tu et al.; this sensor showed enhanced photocurrent signals and allowed glutathione (GSH) detection by optical excitation at 380 nm and H_2O_2 detection by virtue of FeTPPS featuring a molecular structure similar to that of peroxides [10]. In 2012, a PEC sensor was developed by Zhao et al. using graphene and CdS on ITO; in this sensor, graphene, which exhibits excellent charge transport properties, was used as an efficient carrier for spatially separated charges, thereby enhancing and stabilizing the photocurrent and achieving high sensitivity for detecting GSH [11]. Also in 2012, Chen et al. modified an electrode with nanowhiskers of TiO_2 and Pt; these nanowhiskers were made of a composite material with excellent photoelectric properties and used to detect GSH. The nanowhiskers were formed by an inexpensive electrochemical method, showed high sensitivity, and demonstrated good reproducibility [12]. In 2013, Tang et al. prepared several rows of TiO_2 nanowires on the substrate of IrO_2 and modified the TiO_2 nanowire surface with heme, thus forming a combined type biosensor that could be used detecting detect GSH; this sensor showed good adsorptivity, improved sensitivity, and enhanced photocurrent signals [13]. In 2015, Kang et al. developed a PEC sensor with reduced graphene and ZnO nanorods on the FTO substrate; photogenerated electron–hole pair separation efficiency and sensor sensitivity increased because the energy band of the reduced graphene layer matched that of ZnO [14].

The number of cancer cases has continually increased over the last few years, and the causes of cancers vary from one person to another. Thus, the pathomechanisms of cancer are very complex, and cancer detection is often difficult. Cancer diagnosis usually made through clinical examination [15], immunoassay [16], pathological examination [17], fluorescence in situ hybridization (FISH) with DNA sequencing [18], nucleotide sequencing [19], and other related methods. Despite their many benefits, however, these methods present a number of limitations, including poor detection accuracy, time consumption, difficult operation, and high cost, among others. Thus, ZnO/ Cu_2O with excellent properties have been developed for cancer detection ; Cu_2O has a small band gap of roughly 2.0 eV and a suitable conduction band that gives it efficient visible light absorption [20–23]. Furthermore, copper is naturally abundant, and photoelectrodes offer potential competitiveness over other semiconductors [24]. ZnO/ Cu_2O heterostructures can also be applied to solar cells, LEDs, sensors, etc [1, 25, 26]. In the past, multi-spectral imaging combined with monad biochips was applied to develop multi-spectral microscope systems, successfully promoting studies on bladder cancer staging [27]. Using nanoimprint lithography, the hydrothermal method, and RF magnetron sputtering, a novel p-n heterojunction core-shell $\text{Cu}_2\text{O}/\text{ZnO}$ structure was developed [28]. It's expected that, using the biochip integrating the biomedical detection and the fabrication of semiconductor components, as well as the synthesis of semiconductor material and the fabrication of micro/nanostructure, a low-cost and simple biosensor will be developed for faster response, and a functional semiconductor nano-biosensor will be provided with P-N semiconductor heterostructure; and, using PEC properties, cancer cells will be detected, even without extra bias voltage, avoiding the inference of background noise in the detection signal, and increasing the sensor sensitivity.

2. Studied samples and empirical approach

2.1 Material synthesis, component fabrication, and cancer cell culture

The flow chart of the ZnO/ Cu_2O PEC biosensor is given in Fig. 1. First, a $2\text{cm} \times 2\text{cm}$ ITO glass substrate was prepared. After electrochemical deposition of Cu_2O , the electrode surface was placed upward, and the ITO glass substrate was separately rinsed with acetone, methanol, and de-ionized water to reduce impurities (e.g., dust, oil stain, residues after ITO plating, etc.) on the electrode surface. This step prevented contamination of the substrate during Cu_2O deposition and improved the stability of film growth. The Cu_2O film was formed by electrochemical deposition. The electrolyte was made of 0.4M CuSO_4 and 85% lactic acid.

pH was regulated by adjusting the concentration of NaOH. Three electrolytes with different pH were used to determine the photo-chemical response of the biosensor chip. The ZnO seed layer was produced by the spin-coating method. An appropriate amount of the ZnO seed solution was first dripped onto the ITO/Cu₂O substrate. Spin-coating followed in two-stages: (1) place still (0 rpm) for 10 seconds, ensuring the Zn(CH₃COO)₂ coating on the substrate; (2) 15 cycles of repeated spinning (3000 rpm × 30 s) to throw off excess seed solution and form a flat film. After spin-coating, thermal annealing for 30 min was performed at 350 °C under atmospheric conditions to convert Zn(CH₃COO)₂ into ZnO seeds for subsequent growth of ZnO nanorods [29]. Thus, preparation of the ZnO seed layer was completed. ZnO nanorods are prepared by the hydrothermal method. Equal molar volumes of zinc nitrate and hexamethylenediamine were poured and mixed with de-ionized water using a magnetic stirrer and evenly dispersed by ultrasonication to produce an aqueous solution. ZnO seed layer on ITO/Cu₂O substrate obliquely immersed in the aqueous solution that zinc nitrate and hexamethylenediamine are mixed in equal volumes, is placed into an oven at 90°C for 2h continuous growth. Finally, the specimen was taken out, rinsed three times with de-ionized water, and placed in an oven at 80 °C to produce the ZnO nanorods.

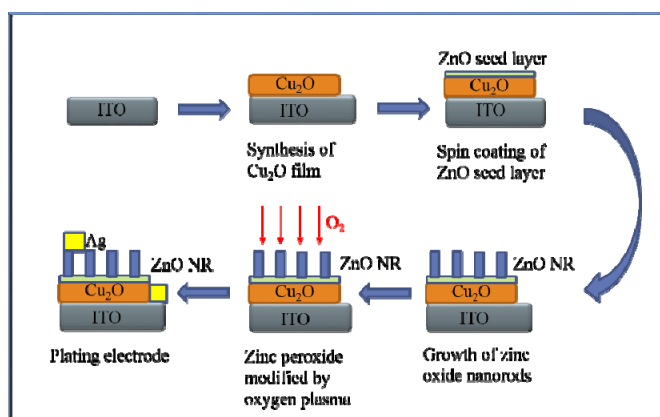


Fig. 1. Fabrication flow chart of the ZnO/Cu₂O photoelectrochemical biosensor.

In water, Zn²⁺ is dissociated from zinc nitrate and NH₃ is released from HMT, thus facilitating the dissociation of OH⁻ from water, promoting the formation of ZnO from Zn²⁺ in reactions, and allowing the growth of ZnO nanorods on the seed layer [30]. Using the plasma oxidation system with oxygen as the working gas, the surface of the specimen prepared as described above was modified to eliminate all of the defects of the ZnO surface and improve the optical and structural properties of ZnO [7]. The system, characterized by the plasma oxidation process of low density and high uniformity and consisting of a single gas pipeline and a cylindrical cavity, places the specimen in the cylindrical cavity, gets the cavity in low vacuum by mechanical pumping, completes the plasma modification on the biosensor surface in the plasma oxidation process, and finally prepares the silver electrode via e-gun vapor deposition system. A schematic of the biosensor is shown in Fig. 2(a). SEM images are provided in Fig. 2(b). Measurements indicate that the ZnO nanorods are straight and vertical with a height of approximately 2 μm and that the Cu₂O film thickness varies with the deposition time within the range of 700 nm – 1 μm. The esophageal cancer cell strains, their culture, and invasiveness are detailed in the Appendix (A1).

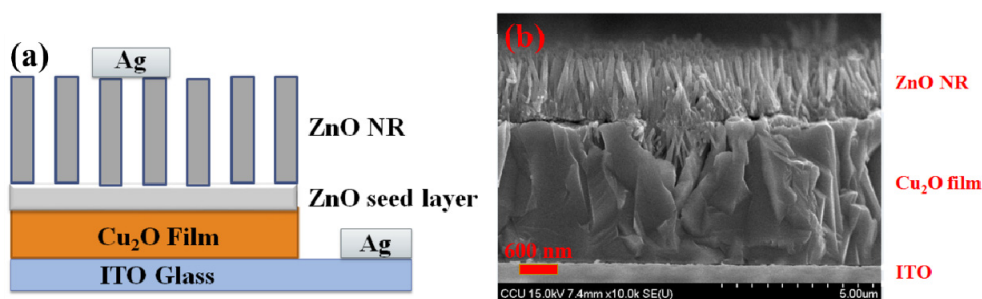


Fig. 2. (a) Schematic diagram and (b) cross-sectional SEM image of the ZnO/Cu₂O photoelectrochemical biosensor.

2.2 Micro-Raman spectroscopy

Figure 3 shows the Raman spectra of the four kinds of ECCs (OE21, OE21-1, CE81T, and CE81T-4). Micro-Raman spectrometer (Renishaw, Invia 1000 system) was used to measure signals with 633 nm laser wavelength, 8.6 mW average power, 40 × magnification, and 4-5 μm spot size. In the process of measurement, the sampled cells and the buffer were mixed and dropped on the slides, and then 70 nm nanogold particles were mixed with the solution. The nanogold particles may enhance the gain of plasma on the surface, thereby increasing the signal intensity of Raman spectroscopy [31–34]. In the Raman spectra of OE21 and OE21-1, the numerical value behind the symbol of “dash” (–) represents the invasiveness of cancer cells; higher the value indicates higher invasiveness. The results showed considerable Raman peak shifts from 1321 cm^{−1} to 1452, 1540, 1580, and 1655 cm^{−1}, which are attributed to lipids, amino acids, CH₂, nuclear acids, and tryptophan, and amide C = O; in addition, the corresponding signals became stronger with the increase in invasiveness [31–34]. Moreover, the spectra of CE81T and CE81T-4 showed the similar results. The above results indicated that OE21-1 and CE81T-4 were more invasive than OE21 and CE81T.

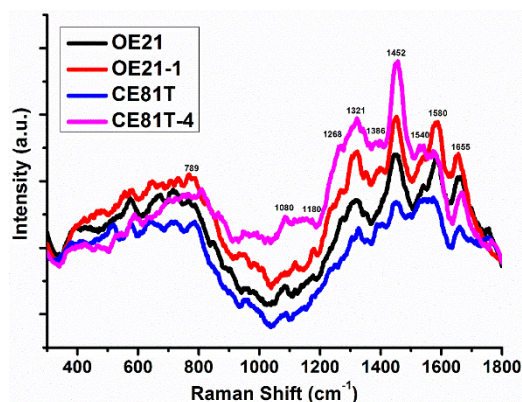


Fig. 3. Raman spectra of the four kinds of ECCs (OE21, OE21-1, CE81T, and CE81T-4).

2.3 Hyper-spectral imaging microscopy (HSIM)

Esophageal cancer presents a spectrum of different diatheses. A precise assessment for individualized treatment depends on the accuracy of the initial diagnosis. Detection relies on comprehensive and accurate white-light cystoscopy. In addition to its invasive nature and the potential risks related to the method, white-light cystoscopy has limitations, including difficulties in flat lesion detection, precise tumor delineation to enable complete resection, inflammation and malignancy differentiation, and grade and stage determination. The resolution of these problems depends on the surgeon's ability and experience with available

technology for visualization and resection. In this study, we used hyper-spectral imaging technology combined with phase contrast microscopy to analyze ECCs at various stages using a single-cell array chip. We found from the spectral characteristics of single cell that the cell spectra at the different cancer stages demonstrate a change in the cell's composition. The hyper-spectral imaging microscopic analysis and invasiveness of esophageal cancer cells are detailed in the appendix.

Polydimethylsiloxane (PDMS) production and microfluidics design with biological compatibility were used along with the hole array structure to position cells without destroying them for further detection and analysis. A microfluidics single-cell array chip designed in this study is shown in Fig. 11(a) in the Appendix. Fabrication of microfluidic chips with microwells are detailed in the appendix (A2.2). The total width of the flow channel was 15 mm, with height and length of 160 μm and 65 mm, respectively. Each flow channel has 10×10 hole arrays. To obtain cells in 10 μm to 15 μm array size, the diameter of the hole was designed as 20 μm , and the diameter of the inlet and outlet of the chip was 3 mm. A syringe pump was used to inject the cell suspension into the single-cell array chip. Phase-contrast microscope images of the single-cell array chip after injection with the four types of ECCs are shown in Fig. 4. Cell images of OE21, OE21-1, CE81T, and CE81T-4 are shown in Figs. 4(a) to 4(d), respectively. The cytoplasm is the white round region, and the nucleus at the center of each cell was darkly stained. The differences between the cancer stages of the four types of ECCs could not be distinguished under the phase-contrast microscope before this experiment began.

The HSIM calculation results of the average spectra of OE21, OE21-1, CE81T, and CE81T-4 cells without any stains are shown in Fig. 5. Each average spectrum was calculated from the amount of data at 400 points (20×20 pixels), taken from 20 cells. The average spectral transmittance increases in the following order: OE21, OE21-1, CE81T, and CE81T-4, because the changes caused by cancer cell progression altered the structure of the cells. More advanced stages of cancer in human ECCs have larger nuclear units [27]. The nucleus, which contains DNA and protein, has lower transmittance than the cytoplasm.

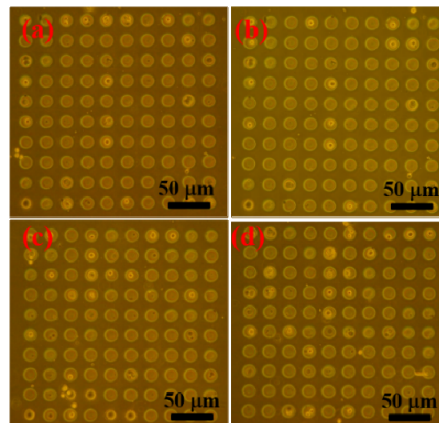


Fig. 4. Single-cell array chip images of the four types of ECCs taken by phase-contrast microscopy: (a) OE21, (b) OE21-1, (c) CE81T, and (d) CE81T-4.

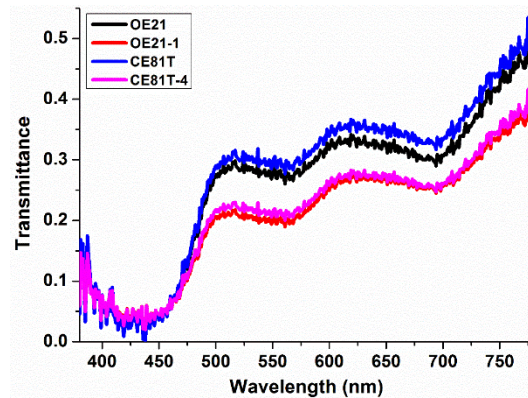


Fig. 5. Average spectra of OE21, OE21-1, CE81T, and CE81T-4.

3. Experimental results and discussion

3.1 XRD results

Figure 6 shows the XRD results of the Sample ITO, Sample ZnO-NR/ITO, and Samples Cu_2O (PH10), Cu_2O (PH11) and Cu_2O (PH12) as the ZnO-NR/ Cu_2O /ITO structures with the pH of 10, 11 and 12 growth condition, respectively. In Fig. 3, peaks at 2θ of 29.6° , 36.5° , 42° , 61.5° , and 73.7° reflect the different planes of Cu_2O at (110), (111), (200), (220), and (311), respectively; these findings are comparable with those in the JCPDS card. At 2θ of 30.4° , 31° , 34.4° , 47° , 56° , 62° , and 67° , the ZnO growth in the (100), (002), (101), (102), (110), (103), (112), and (201) directions relative to the crystallized surface structure may be observed [35, 36]. The diffraction data indicates the crystal phase and average coherence length in the various directions within the crystal. The XRD results of the specimens in Fig. 6, namely, Cu_2O (PH10), Cu_2O (PH11), and Cu_2O (PH12), indicate that increasing the pH of the depositional environment helps gradually increase the signal strength in the preferred direction (111) of Cu_2O , but with the signal strength in the direction (200) decreasing little by little, primarily because of the absorbing lattice plane having the strongest photo-current response of Cu_2O in the direction (111) [37], and a conclusion is drawn that, the higher the pH of the depositional environment, the better crystallinity in the direction (111) plane made with better photo-current response of samples.

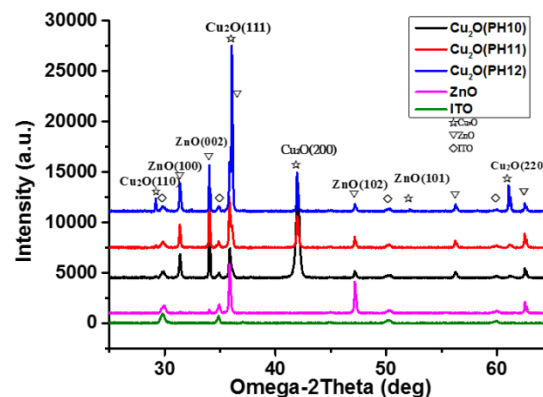


Fig. 6. XRD results of the Sample ITO, Sample ZnO-NR/ITO, and Samples Cu_2O (PH10), Cu_2O (PH11) and Cu_2O (PH12) as the ZnO-NR/ Cu_2O /ITO structures with the pH of 10, 11 and 12 growth condition, respectively.

We also found that the lattice plane of ZnO in the (002) direction exhibits the maximum diffraction peak, indicating the growth of ZnO along the C-axis. In other words, the higher the

peak in the preferred direction (002), the better the overall perpendicularity of ZnO nanorods, which means the joint structures of Cu₂O and ZnO have fewer defects [38]. Thus, the three specimens show good perpendicularity because of thermal annealing of the ZnO seed layer at 350°C [29]; the results also prove that this step is helpful to the vertical growth of ZnO nanorods. Among the specimens [Cu₂O(PH10), Cu₂O(PH11), and Cu₂O(PH12)], Cu₂O(PH12) shows the maximum diffraction peak in the (111) direction of Cu₂O; as such, this specimen has the preferred lattice surface and the best material properties.

3.2 I–V results

Figure 7(a) shows the IV results of the three specimens in the depositional environment with different pH values. The figure shows that the higher the pH of the depositional environment, the better the photo-electric performance is. Cu₂O is sensitive to the pH values of the depositional environment. By taking the advantage of this feature, the preferred growth of the lattice plane is indirectly controlled. By regulating the PH value, the depositional environment usually gives priority to the growth on the lattice plane in the direction (111) or (200), and the plane in the direction (111) is superior in the photo-current response, the light absorption and the preferred growth in the alkaline environment, while the lattice plane in the direction (200) is good in the photo-electric performance and the resistance to corrosion [39]. In the present study, the PEC biosensor chooses the preferred growth of the lattice plane in the direction (111). When the pH is 10, the number of crystals in any other direction is relatively large, likely resulting in more defects and weakening the photo-electric performance. However, as the pH increases, the number of crystals in any other direction gradually decreases with the screening of the preferred effects. With the increasing number of crystals in the preferred direction (111), the photo-electric performance is improved significantly [40, 41]. The measured XRD and I-V properties demonstrate that when the pH is 12, the electrochemically prepared Cu₂O film shows relatively good photo-electric performance. Thus, the pH for the growth of Cu₂O in the depositional environment in the subsequent experiments will be fixed to 12.

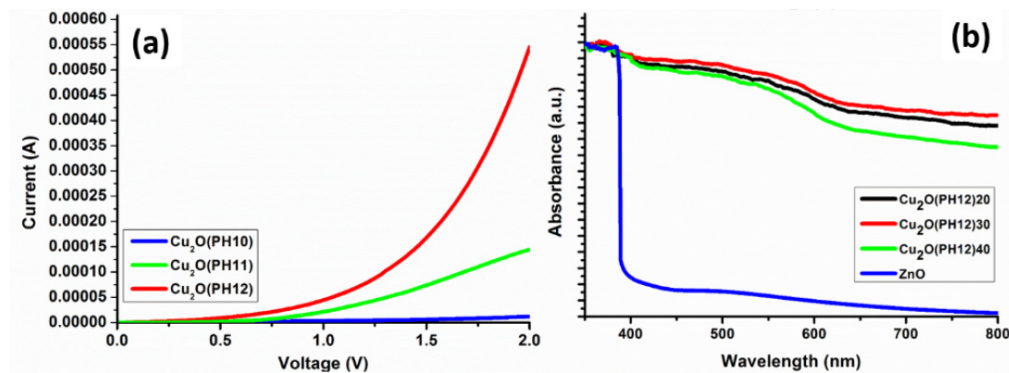


Fig. 7. (a) Characteristic I–V curves of the specimens [Cu₂O(PH10), Cu₂O(PH11), and Cu₂O(PH12)]. (b) Absorption spectra of Cu₂O(PH12) within 20, 30, and 40 min of deposition of Cu₂O.

The light-absorbing layer for every component is principally on the p-type Cu₂O layer [42]. Generally, the thickness of ZnO is 300 nm. Thus, the defects in the material itself may hinder the light absorption of the Cu₂O layer. The thickness of Cu₂O is generally 2 μm, and if the material is too thick, the layer may hinder the transport of the charge carrier and lower the short-circuit current density. Thus, determining the optimal Cu₂O thickness for light absorption by the ultraviolet light-visible light absorption spectrum is necessary. Figure 7(b) presents the results of the specimens: Cu₂O(PH12) and ZnO nanorod at three different points of depositional time by ultraviolet light-visible light absorption spectrum, and the three points

of deposition for 20, 30, and 40 min for the specimens, namely, $\text{Cu}_2\text{O}(\text{PH12})20$, $\text{Cu}_2\text{O}(\text{PH12})30$, and $\text{Cu}_2\text{O}(\text{PH12})40$, respectively.

Figure 7(b) shows that when depositional time increases from 20 min to 30 min, the light absorption relatively enlarges. However, this value declines when the depositional time is 40 min, which is likely caused by the weakening of the light absorption attributed to the thick Cu_2O . Thus, the depositional time for the growth of Cu_2O in the subsequent experiments will be set to 30 min. The figure also indicates that the heterogeneous joint structure of $\text{Cu}_2\text{O}/\text{ZnO}$ has the strongest absorption in the visible light range. However, relative to other research findings, the specimens also exhibit stronger light absorption [1]. Figures 8(a)-8(f) illustrate the vertical and sectional views of n-ZnO/p- Cu_2O at different depositional times by SEM. The difference between various depositional time points may directly affect the thickness of the Cu_2O film. The longer the deposition time, the thicker the film is.

The growth of the Cu_2O film has the following same parameters as 0.45 V voltage, a pH of 12, and temperature at 60 °C. Figures 8(b), 8(d), and 8(f) show that after the growth of the Cu_2O film for 20, 30, 40 min, the film thickness is 754, 873, 1770 nm, respectively. However, the growth of ZnO nanorods at different time points has the same parameters with the length of approximately 2 μm and considerable perpendicularity.

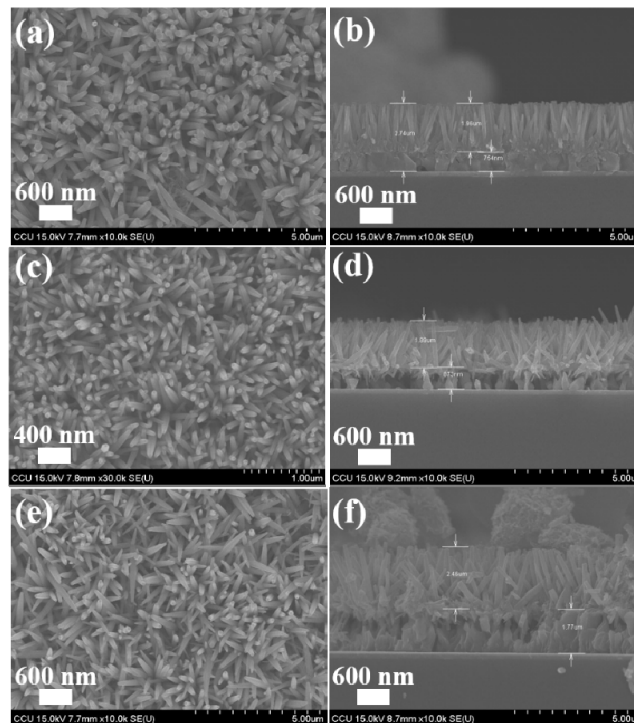


Fig. 8. Vertical/sectional views of n-ZnO/p- Cu_2O under SEM at different time points of Cu_2O film deposition and after 2 h of growth of fixed ZnO nanorods. (a) Vertical and (b) sectional views of the Cu_2O film after 20 min growth. (c) Vertical and (d) sectional views of the Cu_2O film after 30 min growth. (e) Vertical and (f) sectional views of the Cu_2O film after 40 min growth under SEM.

3.3 Photo-current responses of esophageal cancer cells

In general, glutathione in the cells exists in the forms of the reduced GSH and oxidized GSSG. For normal cells, approximately 90% of glutathione exist in the form of GSH in the cytoplasm. However, only 10% of glutathione are in mitochondria, except for a small part in endoplasmic reticulum, and the ratio of GSH to GSSG in cells is greater than 10:1 [43]. The

unbalanced redox homeostasis under the oxidative stress inside the cancer cells is more serious than that in the normal cells, therefore, when the cells begin carcinogenesis, the ratio of GSH to GSSG may decline, which means that the GSSG content may rise under the oxidative stress (OS) and the GSH content may decline on the contrary [43]. Thus, when the biochip is used for detecting the cancer cells in principle because of the higher GSSG content under the oxidative stress inside the cancer cells, the biochip produces a gain in the photo-current. The more severe the carcinogenesis, the larger the oxidative stress inside the cancer cells is, which indicates higher GSSG content. The GSH/GSSG physiological mechanisms of esophageal cancer cells are detailed in the appendix (A3.1).

The experimental method is described as follows: First, the cancer cells are sorted, collected, and counted, and the volume of the test solution is determined to ensure the consistency in the number of cancer cells. We detected the GSSH concentration outside cells. The concentration of GSSH is 100 μM in PBS solution. A photo-electric performance analyzer is used to measure and observe the switching behaviors of the original components, as well as those the components after PBS and four kinds of cancer cells dropped in with the experimental results in Fig. 9. The photo-current behaviors of the four types of cancer cells, as well as the tendencies of the carcinogenesis and photo-current, are clearly observed. The more severe the carcinogenesis, the higher the GSSG content is with the larger photo-current.

Figure 9(a) demonstrates that the changes of the biosensor are divided into three stages: (1) At the first stage, $\text{ZnO}/\text{Cu}_2\text{O}$, which is excited by light, produces a significant number of electron-hole pairs, and the measured current by the photo-electric performance analyzer becomes larger, indicating that this set of components is responsive to light. (2) At the second stage, the photo-current increases more slowly, which is related to the presence of the electron-hole pairs; the components are excited by light to produce electron-hole pairs and affected by the electron-hole pairs. The bonding between the pairs disappears. Thus, the photo-current produced by the components is a final result of the production and re-bonding of the electron-hole pairs. In principle, the photo-current climbs positively with the higher production rate of the electron-hole pairs than the re-bonding rate.

The photo-current after PBS saline reduces primarily because the cations in the PBS absorb and bond electrons, which is helpful for the transport of electrons inside the components. The photo-current increases after OE21 esophageal cancer cells are dripped in chip because the cancer cells contain GSSG and the content of GSSG depends on the carcinogenesis and the number of cancer cells. GSSG can facilitate the electron transport inside the components, thus enlarging the photo-current. (3) After the light is turned off at the third stage, no electron-hole pairs are produced because of the disappearance of the light source. At the same time, the bonding of electron-hole pairs being affected by the survival time begins to disappear again, thus, when the electron-hole pairs completely disappear, no photo-current may be produced. However, the decline rate of the photo-current of the components being affected by cancer cells PBS or OE21 is low, so in the fall of the current cancer cells PBS or OE21 are still helpful for the electron transport. In the subsequent experiments, cancer cells involved in different carcinogenesis are sampled from various races. The observed behaviors of the components are basically consistent with the description above, and the results are shown in Fig. 9 (b). Figures 9(a) and 9(b) show that the measured photo-current values of OE21, OE21-1, CE81T2-1, and CE81T2-4 are 158%, 223%, 78%, and 308% higher than those measured by an “empty” biosensor, respectively. The results are consistent with the results of the carcinogenesis of esophageal cancer cell strains: the carcinogenesis of OE21-1 is more serious than that of OE21 [44], the measured value by the biosensor also exhibits certain tendency, and the carcinogenesis of CT81T2-4 is more serious than CT81T2-1 [45]. The measured current values determined by the biosensor demonstrate a trend, and the results validate the hypotheses of the present study: the more severe the carcinogenesis, the higher the concentration of GSSG inside the cancer cells and the larger photo-current measured by the biosensor.

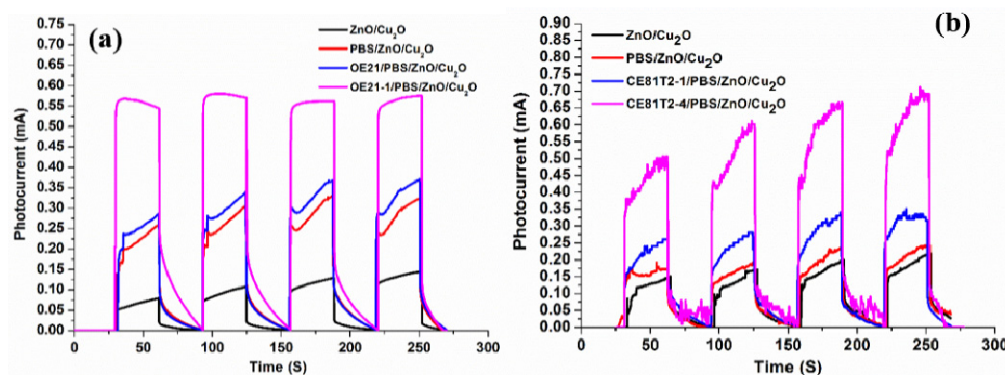


Fig. 9. Photo-current responses of the biosensor to the following cells: (a) Esophageal cancer cells (OE21) involved in different cancer stages of Caucasian and (b) Esophageal cancer cells (CE81T2) involved in different cancer stages of Asian.

4. Conclusions

In the present study, electrochemical deposition is adopted to grow p-type Cu_2O films. X-ray monocrystalline diffraction analysis, photo-electric performance analysis, UV-vis spectrometry, and sweep electron microscopy, among others, are used for detection. The results show that the Cu_2O film obtained at pH 12 after growth for 30 min produces Cu_2O crystals with the preferred lattice plane (111), forming a film with excellent photo-electric performance. The prepared Cu_2O crystals form highly regular and complete lattices. The hydrothermal method is adopted for the growth of the n-type ZnO nanorods supplemented by the ZnO seed layer and the thermal annealing, thus improving the perpendicularity and integrity of the crystalline form. By employing the steps mentioned earlier, the PEC biosensor, which exhibits considerable photo-current responses, was successfully fabricated. Four strains of esophageal cancer cells involved in different cancer stages were sampled from two various races and placed in the sensor for photo-chemical measurement. The experiment proves that the biosensor may produce stronger photo-current signals for more serious esophageal cancer cells. The severer the carcinogenesis is, the higher content of GSSG is, with the larger photo-current; GSSG helps increase the photo-current in the electron transport process.

Appendix

A1.1 Esophageal cancer cell strains and their culture

The present study investigated four esophageal cancer cell (ECC) strains, namely, OE21, OE21-1, CE81T2-1, and CE81T2-4, which were provided by the Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Medical University Hospital. Among the strains, OE21 and OE21-1, which exhibited the highest invasiveness, were also called JROECL21. In 1993, squamous cell carcinoma strains were sampled from the middle esophagus of a 74-year-old male patient with pathologically staged IIA(UICC), and the cancer cells were moderately differentiated, belonging to HLA-A, HLA-B, and HLA-C(MHC I) [44]. CE81T-1, which exhibited the lowest invasiveness, and CE81T-4, which showed the highest invasiveness, were squamous ECC strains sampled from the middle esophagus of a 57-year-old Taiwanese male patient in 1981, presenting highly differentiated squamous cell carcinoma [45]. The RPMI culture medium was used for normal esophageal cells OE21 from a white man, as well as for cancer cells OE21-1 (exhibiting the higher invasiveness in whites) and CE81T2-1 (exhibiting the lowest invasiveness in yellows); DMEM was used as culture medium for cancer cells CE81T2-4 (exhibiting the highest invasiveness in yellows). Furthermore, 10% fetal bovine serum (FBS, Gibco, Grand Island, USA) and 1% penicillin

(Gibco, Grand Island, USA) were added to the two culture media. The sampled cells were cultured on Petri dishes (Falcon, Franklin Lakes, NJ, USA) and placed in an incubator at 37 °C containing 50% carbon dioxide. Culture media were replenished, or cells in Petri dishes were sorted, every two days as appropriate. In addition, phosphate buffered saline (PBS, Biochrome, pH 7.4) was used for rinsing while sorting the cells on Petri dishes. Then, 0.25% trypsin and 0.02% EDTA (Sigma, USA) were added to the cell mixture after 5 min to obtain a cell suspension.

A1.2 Selection of invasion cells by transwell invasion chamber sophageal cancer cell strains and their culture

Subpopulations from the CE81T ECC line were selected using the membrane invasion culture system (MICS) or BD BioCoat Matrigel Invasion Chamber (MA, USA). Briefly, cells were suspended in DMEM containing 10% FBS and seeded into the wells. After incubation at 37 °C for 72 h, the inserts were removed. The cells that had invaded the membranes and had attached to the lower-chamber compartments were harvested and allowed to proliferate for a second round of selection. For MICS selection, the sublines of the first-round selection in the upper and lower well chambers were designated as CE81T1-0 and CE81T1-1, respectively; the sublines from the second, third, and fourth rounds of selection were designated as CE81T1-2, CE81T1-3, and CE81T1-4, respectively. The parental line in the first series was designated CE81T1. For BD BioCoat Matrigel Invasion Chamber selection, the sublines of the first-round selection in the upper and lower well chambers were designated CE81T2-0 and CE81T2-1, respectively, and the sublines from the second, third, and fourth rounds of selection were designated CE81T2-2, CE81T2-3, and CE81T2-4, respectively. The parental line in the second series was designated CE81T2 [46].

A1.3 In vitro invasion assay

MICS was used to measure the invasion capacity of each cell line. Assays were performed using polycarbonate membranes (Falcon HTS Fluoro Blok insert, BDBiosciences, Franklin Lakes, NJ, USA). Invasion assay was performed using membranes with uniformly-coated reconstituted basement gel (Matrigel, BD Biosciences, Bedford, MA, USA). The insert was placed in a 24-well culture dish (Falcon) containing DMEM and 10% NuSerum (BD Biosciences). In each well, 5×10^4 cells were resuspended in DMEM containing 10% NuSerum, and then seeded into the upper wells of the chamber. After 36 h of incubation at 37 °C, cells that had migrated or had invaded the membrane were stained with the fluorogenic compound 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA). Invading cells were counted manually using three random microscopic fields per well. The DAPI fluorescence of nuclei was visualized by excitation at 330–385 nm with a 420 nm barrier filter. Images were captured using a Nikon inverted fluorescence microscope (Nikon ECLIPSE TE300, Tokyo, Japan) with attached camera at $\times 100$ magnification and processed using ImagePro PlusVersion 5.0 software (Media Cybernetics, MD, USA). Experiments were repeated in triplicate.

A2.1 Estimation processes of HSIM

The estimation processes of the average spectra of CE81T, and CE81T-4 cells with HSIM data are illustrated in Fig. 10. HSIM technology was used to obtain the spectrum of each image element of the single-cell array. First, the spectra of the 24 Macbeth color checkers are measured by a spectrophotometer (Konica Minolta CS1000A) under the illumination of a particular uniform artificial light, and the reflection spectrum of each color checker in the visible light region (380nm to 780nm) is obtained. These spectra are arrayed as a matrix, $[D]_{401 \times 24}$, the rows of which are the intensities of the wavelengths at 1 nm intervals, and the columns of which are the numbers of the color checkers. By determining the eigen-system and applying the principal component analysis (PCA), six eigen vectors that make the greatest

contribution are selected to be the basis of the spectral estimation, and arrayed as a matrix $[E]_{6 \times 401}$. The corresponding eigen values of these six eigen vectors $[\alpha]_{6 \times 24}$ can be determined as follows:

$$[\alpha]^T = [D]^T \text{pinv}[E] \quad (1)$$

where “pinv” denotes the pseudoinverse of the matrix. Simultaneously, the color checkers are captured by a digital camera under the same illumination condition, for which the output format is sRGB (JPEG image files). The red, green and blue values (from 0 to 255) of each color checker's image are obtained using computer programs, and are then plotted on a scale of R_{srgb} , G_{srgb} and B_{srgb} (from 0 to 1). These RGB values can be transferred into CIE XYZ tristimulus values by the following formula:

$$\begin{bmatrix} X \\ Y \\ Z \end{bmatrix} = [T] \begin{bmatrix} f(R_{srgb}) \\ f(G_{srgb}) \\ f(B_{srgb}) \end{bmatrix} \quad (2)$$

where

$$[T] = \begin{bmatrix} 0.4124 & 0.3576 & 0.1805 \\ 0.2126 & 0.7152 & 0.0722 \\ 0.0193 & 0.1192 & 0.9505 \end{bmatrix} \quad (3)$$

$$f(n) = \begin{cases} \left(\frac{n+0.055}{1.055} \right)^{2.4}, & n > 0.04045 \\ \left(\frac{n}{12.92} \right), & \text{otherwise} \end{cases} \quad (4)$$

Due to the reference white of the sRGB color space being illuminated by a CIE standard light source D65, which is different from the artificial light used for measuring the spectra of the color checkers, these RGB values are corrected for chromatic adaptation by applying CMCCAT2000. Taking the accuracy of spectral estimation into account, color correction of the camera is also required. The reflection spectra measured by the spectrophotometer are transferred into CIE XYZ tristimulus values by using Eqs. 5 to 8. In these calculations, $S(\lambda)$ is the relative spectral power distribution of the artificial light, $R(\lambda)$ is the spectral reflectance of the respective color checker $\bar{x}(\lambda)$, $\bar{y}(\lambda)$, and $\bar{z}(\lambda)$ are the color matching functions,

$$X = k \int_{380nm}^{780nm} S(\lambda) R(\lambda) \bar{x}(\lambda) d\lambda \quad (5)$$

$$Y = k \int_{380nm}^{780nm} S(\lambda) R(\lambda) \bar{y}(\lambda) d\lambda \quad (6)$$

$$Z = k \int_{380nm}^{780nm} S(\lambda) R(\lambda) \bar{z}(\lambda) d\lambda \quad (7)$$

where

$$k = 100 / \int_{380nm}^{780nm} S(\lambda) \bar{y}(\lambda) d\lambda \quad (8)$$

After the chromatic adaptation transform, the RGB values corresponding to the new XYZ values are calculated by the inverse procedures of Eqs. 2 to 4, then set as standard matrix $[A]$. The color relationship between the spectrophotometer and the camera is found by using the third-order polynomial regression for the red, green and blue components separately, and the regression matrix $[C]$ is determined as follows:

$$[C] = [A] \text{pinv}[F] \quad (9)$$

Where

$$[F] = [1, R, G, B, RG, GB, BR, R^2, G^2, B^2, RGB, R^3, G^3, B^3, RG^2, RB^2, GR^2, GB^2, BR^2, BG^2]^T \quad (10)$$

and “R”, “G”, and “B” are the respective RGB values of the color checkers captured by the camera. The corrected RGB values are obtained from Eq. (11). Here $[K]$ presents the RGB values captured from any image that expanded into a format such as the original matrix $[F]$. and arrayed as a matrix, $[\beta]$.

$$[\text{Corrected RGB}] = [C][K] \quad (11)$$

Finally, a transform matrix $[M]$ between the spectrophotometer and the camera is obtained as follows:

$$[M] = [\alpha] \text{pinv}[\beta] \quad (12)$$

For each pixel in any image captured by the camera, the RGB values multiplied by the regression matrix $[C]$ and the corresponding XYZ values are calculated using Eqs. 2 to 4. The estimated spectra in the visible light region (380nm to 780nm) are obtained by

$$[Spectra]_{380-780nm} = [E][M] \begin{bmatrix} X \\ Y \\ Z \end{bmatrix} \quad (13)$$

Esophageal cancer presents a spectrum of different diatheses. A precise assessment for individualized treatment depends on the accuracy of the initial diagnosis. Detection relies on comprehensive and accurate white-light cystoscopy. In addition to its invasive nature and the potential risks related to the method, white-light cystoscopy has limitations, including difficulties in flat lesion detection, precise tumor delineation to enable complete resection, inflammation and malignancy differentiation, and grade and stage determination. The resolution of these problems depends on the surgeon's ability and experience with available technology for visualization and resection. In this study, we used multi-spectral imaging technology combined with phase contrast microscopy to analyze ECCs at various stages using a single-cell array chip. We found from the spectral characteristics of single cell that the cell spectra at the different cancer stages demonstrate a change in the cell's composition.

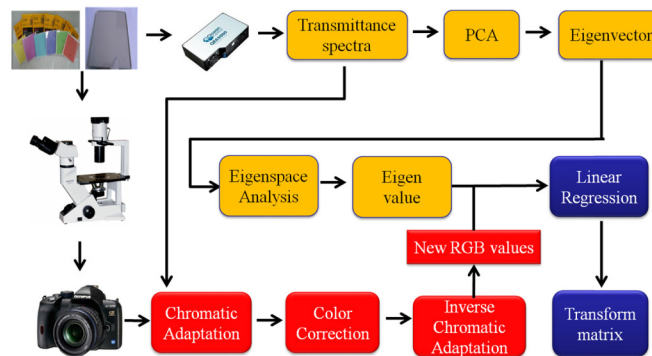


Fig. 10. Schematic diagram of the proposed method used in estimating the spectral transmittance of each pixel of an image using a DSLR camera.

A2.2 Fabrication of microfluidic chips with microwells

A biocompatible material, PDMS, was adopted for single-cell-based arrays in the microfluidic chip, as illustrated in Fig. 11(a). The main channel, formed on the top PDMS layer, is 15 mm wide, 160 μm in height and 65 mm long. The main channel is divided into eight microchannels, each 1 mm wide and 45 mm long, at the center region. Each microchannel contains fifteen 10×10 microwells, 20 μm or 30 μm in diameter and 20 μm deep, on the bottom PDMS layer. The mold masters were fabricated by spinning SU-8 (SU-8 50, MicroChem Corp., Newton, MA, USA) on a silicon wafer to define the microwells and microchannel, respectively. The mold master of the microfluidic channels (around 160 μm in height) was fabricated by spinning SU-8 at 500 rpm for 20 s and then at 800 rpm for 35 s on the silicon wafer. The resist was soft baked on a hotplate at 65 $^{\circ}\text{C}$ for 10 min and then at 95 $^{\circ}\text{C}$ for 30 min. The resist was then allowed to cool to room temperature. The SU-8 was exposed to ultraviolet (UV) radiation at a dose of 200 mJ/cm^2 . The post-exposure baking was done at 65 $^{\circ}\text{C}$ for 3 min and then at 95 $^{\circ}\text{C}$ for 10 min. The exposed samples were developed with SU-8 developer for 5 min. The mold master of the microwells (around 20 μm in height) was fabricated by spinning SU-8 at 500 rpm for 20 s and then at 4,500 rpm for 35 s on a silicon wafer. The resist was developed with SU-8 developer for about 2 min after baking and exposure to UV radiation under the conditions mentioned above. PDMS prepolymer mixture (Sylgard-184 Silicone Elastomer Kit, Dow Corning, Midland, MI, USA) was poured and cured on the mold masters to replicate the patterned structures. Scanning electron microscopy (SEM) images of the SU-8 mold with microwells on the silicon wafer and PDMS replica are shown in Fig. 11(b). After peeling off the PDMS replica with the microchannel, the inlet and outlet ports were made by a puncher. The two PDMS replicas were bonded after treatment with oxygen plasma in an O₂ plasma cleaner (model PDC-32G, Harrick Plasma Corp., Ithaca, NY, USA). A photograph of the completed microfluidic chip with tubing is shown in Fig. 11(c).

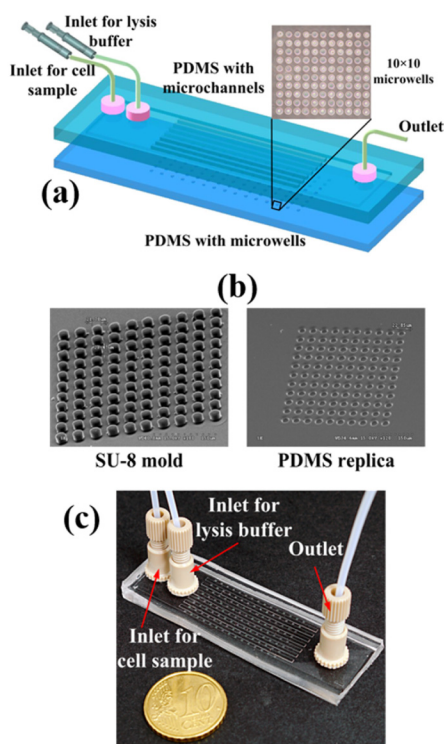


Fig. 11. (a) Schematic diagram of the proposed microfluidic chip for single-cell-based microarrays. (b) SEM micrographs of the SU-8 mold on the silicon wafer and PDMS replica. (c) Photograph of the completed microfluidic chip with tubing.

A3.1 GSH/GSSG physiological mechanism

Glutathione (GSH) widely exists in small-molecule tripeptide compounds within cells involved in intracellular amino acid transport, glycometabolism, and DNA synthesis and regulation. GSH plays an important role in inhibiting exogenous toxicants and oxygen-mediated radical injuries, regulating the body's immunological function, maintaining the structures and functions of cellular proteins, restraining the apoptosis, etc. Cell injuries induced by oxygen free-radicals and lipid peroxidation have been investigated for years, and the oxidation–reduction degree of GSH is an important indicator in the evaluation.

A3.2 General physiological characteristics of GSH

GSH is composed of three kinds of amino acids (glutamic acid, cysteine, and glycine), and it is the most abundant thiol-containing compound in liquid form within cells, and the active center of GSH is -SH attached to the α -amino group of cysteine. The concentration of GSH varies among visceral organs; the highest concentration is detected in the liver, followed by the spleen, kidney, lung, brain, heart, pancreas, and bone marrow, and the lowest concentration is in blood. Moreover, different parts of a same organ differ considerably in GSH concentration, and the content of GSH in a same cell varies greatly from one organelle to another. Under the actions of oxidants, GSH is converted to oxidized GSH (GSSG) via GSH-peroxidase (GSH-Px); by contrast, GSSG with hydrogen supplied from NADPH is reduced to GSH under the action of GSH reductase (GSH-Rx). GSH and GSSG form a dynamic equilibrium in the cells with GSSG maintained at 1%–10% of the total GSH, thus constituting an effective antioxidant system. The ratio of GSH to GSSG is high in the physiological state; however, under oxidative stress, GSH is oxidized to GSSG, thereby

decreasing the ratio of GSH to GSSG; thus, this ratio may be used as indicator for evaluating cell injuries induced by lipid peroxidation [47].

A3.3 Mechanisms of GSH inhibiting cell injuries induced by oxygen free-radicals and lipid peroxidation eneral physiological characteristics of GSH

GSH plays an important role in inhibiting oxidative toxicants. On one hand, GSH can bind with molecular toxicants and their metabolites, thus reducing the toxicity. On the other hand, GSH can lower the reoxidation of the toxicants by redox reaction, thereby avoiding thiol-containing enzymes from being activated by heavy metals and oxidants, or restoring the activity by reducing the oxidized thiol-containing enzymes. When generating a large number of free radicals, polyunsaturated fatty acids in cytomembrane are oxidized to lipid-peroxy radicals, causing a series of secondary injuries. GSH can directly inhibit oxygen radicals using the toxic properties of H^+ supply, thereby stopping the chain reaction, and GSH is itself oxidized to GSSG. Similarly, GSH plays an important role in inhibiting oxygen free-radicals and lipid peroxidation and the corresponding apoptosis, necrosis, homeostasis changes, etc. The metabolism of GSH/GSSG in the body has multiple pathways. In an oxidation state, GSH is, on one hand, oxidized to GSSG, as manifested by the ratio of GSH to GSSG, and, on the other hand, GSH binds with exogenous toxicants and their metabolites, ultimately producing mercapturic acid, which is excreted with the urine, resulting in only a reduction in GSH, and probably without significant changes in GSSG, or even with GSSG reduction because of GSH consumption. GSSG cannot only be reduced to GSH but also have a binding reaction via GSH-S-transferase (GST) [48].

A3.4 Correlation between GSH/GSSG proportion and cancer cells

In general, GSH in cells exists in the reduced form (i.e., GSH) and the oxidized form (i.e., GSSG). Normal cells contain about 90% of GSH in cytoplasm and only 10% of GSH in the mitochondria, except for a small concentration in the endoplasmic reticulum; the ratio of GSH to GSSG in cells is greater than 10:1. The oxidation–reduction ratio imbalance in cancer cells under the oxidative stress is greater than that in normal cells, and reactive oxygen species (ROS) may attack the structure of DNA, and thus, the DNA mutation is the key factor for cell carcinoma. In the normal cell metabolism, ROS in cells primarily come from the electron transport chain on the mitochondrial membrane. An appropriate concentration of ROS can be used as intracellular messenger molecules; however, high expression level of ROS in the cells may cause cytotoxicity and even cell death. Thus, the balance between intracellular ROS and antioxidant capacity is significant in normal physiological functions. During carcinogenesis, the ratio of GSH to GSSG in cells decreases; thus, an increase in the GSSG content with a decrease in the GSH content may indicate considerable oxidative stress [43].

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