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# Intracellular ZnO Nanorods Conjugated with Protoporphyrin for Local Mediated Photochemistry and Efficient Treatment of Single Cancer Cell

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**Abstract** ZnO nanorods (NRs) with high surface area to volume ratio and biocompatibility is used as an efficient photosensitizer carrier system and at the same time providing intrinsic white light needed to achieve cancer cell necrosis. In this letter, ZnO nanorods used for the treatment of breast cancer cell (T47D) are presented. To adjust the sample for intracellular experiments, we have grown the ZnO nanorods on the tip of borosilicate glass capillaries (0.5 μm diameter) by aqueous chemical growth technique. The grown ZnO nanorods were conjugated using protoporphyrin dimethyl ester (PPDME), which absorbs the light emitted by the ZnO nanorods. Mechanism of cytotoxicity appears to involve the generation of singlet oxygen inside the cell. The novel findings of cell-localized toxicity indicate a potential application of PPDME-conjugated ZnO NRs in the necrosis of breast cancer cell within few minutes.

**Keywords** ZnO nanorods · Cancer cell necrosis · Photodynamic therapy · Protoporphyrin dimethyl ester

## Introduction

Zinc oxide (ZnO) is gaining much research attention due to many advantageous properties like direct band gap of 3.37 eV and large exciton binding energy of 60 meV at room temperature and deep level defects emissions that cover the whole visible range. In fact, ZnO is one of the very few materials that can emit 'intrinsic' white light. In addition, ZnO nanostructures family is the richest known among all materials so far and the growth of these nanostructures is facilitated by the self organized growth property of this material. It can be grown with good crystalline quality on almost any substrate being crystalline or amorphous [1]. Due to these properties, it is regarded as a very promising material for many photonic devices [1–3] including devices for the treatment of cancer cells [4, 5]. Moreover, ZnO is a bio-safe and bio-compatible material and this makes it an attractive candidate for biomedical applications [6, 7]. Materials when scaled down to the nanoscale realm, unique size-dependent properties of these nanomaterials are manifested.

Moreover, ZnO NRs having a large surface area to volume ratio and can be grown with vertical alignment nanorods makes them also natural wave guiding cavities enhancing the light extraction efficiency in photonic devices [8]. Due to this, ZnO has a potential for a wide range of applications in UV and intrinsic white light–emitting devices. The UV and green emission part of the white light of ZnO can be used to activate some biological process, such as the activation of photosensitizers for photodynamic therapy (PDT) [9–11]. There are few reports dealing with ZnO nanoparticles for bio-medical purposes e.g. PDT [4, 12].

Photodynamic therapy has become an important tool for the treatment of cancer cells [13–18]. As well known photodynamic therapy for cancer cell involves the uptake of photosensitizer by cancer cells followed by exposure to white light. The choice of a suitable irradiation wavelength is one of the basic parameters affecting the efficiency of the PDT. Irradiation with long-wavelength light to protoporphyrin IX

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dimethyl ester (PPDME), which absorbs 630 nm wavelength light, provides low quantum yields for singlet oxygen [19]. Normally the photosensitizer is typically applied to the tumor for 3–6 h before light exposure so that adequate amount of it can penetrate into the tumor. The molecular oxygen in the cell is essential for the PDT, as the phototoxic reaction involves the formation of singlet oxygen in the PDT [20]. The singlet oxygen is produced by the energy transfer between the molecular oxygen and the triplet state of the excited photosensitizer molecules in the cell [21]. This singlet oxygen will cause initial damage to the mitochondria leading to cell necrosis [22]. The cell necrosis due to this damage of mitochondria is still under discussion. However, the penetration of the drug (aminolevulinic acid (ALA)) is the major limiting factor in the PDT [23]. For this reason, there is a demand for new and improved photosensitizer delivery systems for effective treatment modalities.

In this paper, ZnO NRs are suggested and used as efficient carrier of the photosensitizer for cancerous cell treatment. These ZnO nanorods are grown on the tip of a submicrometer tip glass pipette and the photosensitizer was applied to the surface of the grown ZnO nanorods. By mechanical manipulation, the conjugated ZnO NRs were inserted gently inside the cancer cells. The emission of intrinsic white light which sensitize the cancer cells, when excited by UV light is investigated. For this purpose, an inverted fluorescence microscope (ZEISS) was used. The experiments regarding the florescence (Fl) spectra were performed for configurations with bare ZnO NRs and for those with conjugated photosensitizer using an excitation wavelength of 240 nm.

## Experimental Procedure

The main effort has been focused to make the tip geometry small enough. Extremely sharp (sub-micrometer dimensions) and long tips (>10 μm in length) are the basic requirement for the intracellular PDT device in the present work. Such intracellular device should have properties like bending and gentle penetration of the flexible cell membrane, which are provided by ZnO NRs.

Borosilicate glass capillaries (sterile Femtotip® II with tip inner diameter of 0.5 μm, an outer diameter of 0.7 μm, and a length of 49 mm, Eppendorf AG, Hamburg-Germany) were fixed on a flat support in the vacuum chamber of an evaporation system (Satis CR725), so that a thin chromium and silver films (with a thickness of 10 and 125 nm, respectively) were uniformly deposited onto the outer surface of the capillary tips as shown on SEM image in Fig. 1a. On the silver-coated capillary glass tip, we grew hexagonal single crystals of ZnO NRs using a low-temperature method [24, 25]. The morphology and some structural information of ZnO NRs are shown in the SEM

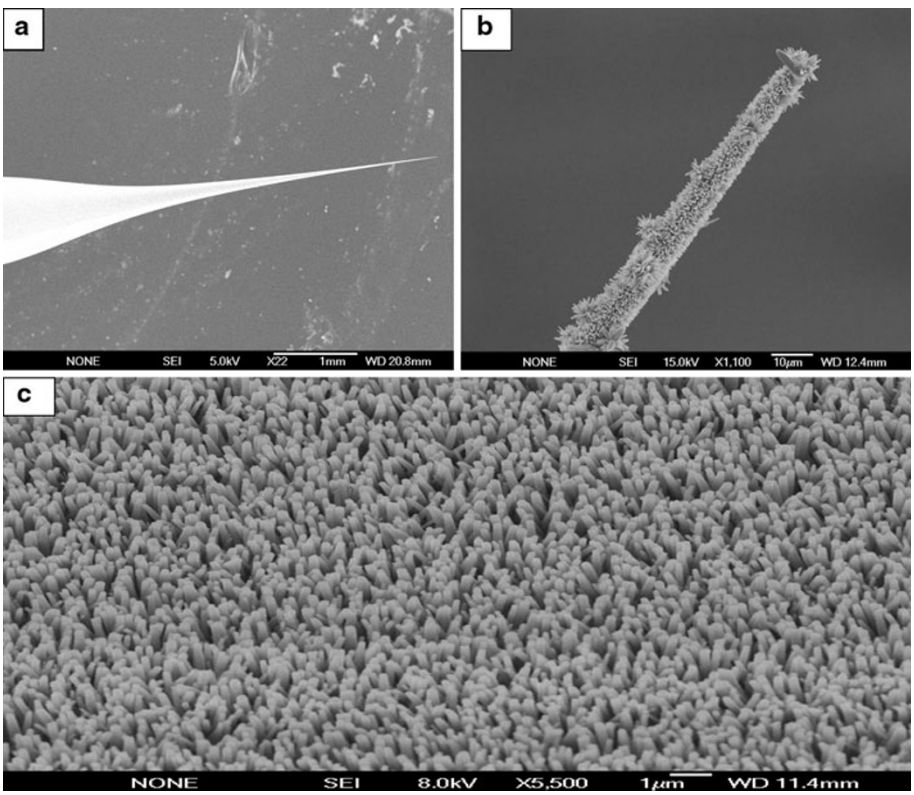


**Fig. 1** SEM images taken for the ZnO nanorods grown at the tip of the present PDT device, in **a** SEM image of a silver-coated tip, in **b** and **c** SEM images of ZnO NRs grown on the silver-coated sub-micrometer tip

images in Fig. 1b, c. The ZnO NRs are 150–170 nm in diameter and around 1 μm in length. The ZnO NRs on the silver-coated capillary glass tip were conjugated with protoporphyrin layer through a manual process. Powdered protoporphyrin was dissolved in methanol and N, N-Dimethylformamide with a ratio of 1:1. The ZnO NRs-coated tips were dipped three times into the prepared solution. After each dip, the tip was allowed to dry at room temperature. A submicrometer glass pipette covered with bare grown ZnO NR was also used as a reference PDT devices to allow the separation of the contribution to fluorescence effect of the NRs and the photosynthesize element. These bare and conjugated ZnO NRs devices were used as local PDT intracellular photosensitizer delivery system for breast cancer treatment. We used breast cancer cells line ATCC (American Type Culture Collection)-HTB-133 designations T47D purchased from LGC Standard AB Borås, Sweden. These cells were first trypsinized. Some of these cells were transferred to a regular Petri dish in the culture medium so that cells can be attached to the bottom of the Petri dish for 12 h, in an incubator at 37°C in $CO_2$ atmosphere.

We have used a technique of intracellular mechanical manipulation of a bare or functionalized the tip of a submicrometer inside single cells [6, 7]. This technique although an invasive of nature, the penetrated cells have shown not to be affected by the invasion. The cancer cells were studied using an inverted fluorescence microscope (ZEISS). Cell necrosis was noted when exposed to the emitted white light.

## Results and Discussion

Borosilicate glass capillaries femtotip II coated with silver is shown in the SEM image in Fig. 1a, while Fig. 1b shows

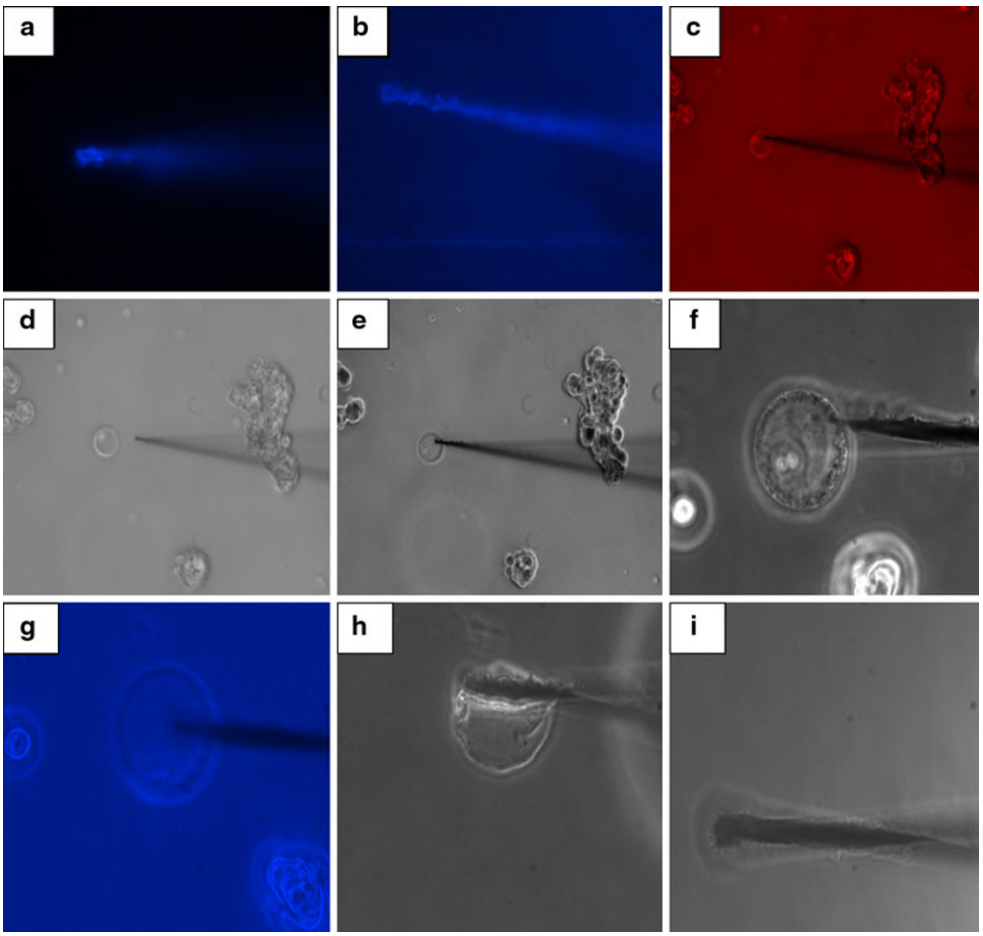


**Fig. 2** Digital images taken during the performance of the intracellular measurements, **a** and **b** show the fluorescence images of the bare and the PPDME-conjugated ZnO NRs, respectively. **c** Image of the PPDME-conjugated ZnO NRs tip inside the cancer cell. **d** Image of the conjugated ZnO NRs tip near the cancer cell. **e** Image of the conjugated ZnO NRs tip inside the cancer cell. In **f** and **g** magnified images of the cancer cell. **h** Shows the cancer cell under necrosis with intracellular penetration of the conjugated ZnO NRs tip. Finally in **i** conjugated ZnO NRs tip after cancer cell necrosis

the tip with the grown ZnO NRs. As can be seen, the ZnO NRs have a hexagonal shape and a uniform density as shown in SEM image of Fig. 1c.

Images taken by a coupled charge camera device (CCD) connected to the inverted microscope are shown in Fig. 2. As a control experiment, the excitation of a bare and conjugated ZnO NRs was first performed. Figure 2a, b shows the fluorescence from the tip of the PDT devices for cases with and without the protoporphyrin, respectively. These photographs were taken when a filter (DAP1) for UV excitation was used. It can be seen that the fluorescence is relatively high using both the bare ZnO and conjugated ZnO nanorods. As mentioned above, vertical nanorods are like natural wave guiding cavities for making the emitted light travels efficiently to the top of the device minimizing partial leakage and thus enhancing the light extraction efficiency from the PDT device [8]. ZnO nanorods with a large surface area to volume ratio are of potential for more drug delivery to the tumor site. Different stages from the start of the mechanical manipulation of the PDT device inside the cancer cell until the cell necrosis are shown in Fig. 2c–i. Images shown in Fig. 2c–g show the tip and the cancer cell in the Petri dish. In the image shown in Fig. 2d, the tip was near the cell but has not yet been inserted inside the cell. Here, the UV light exposure time was 10 min and there was no cell necrosis. It means neither the PPDME nor the molecular oxygen was available inside the cell to facilitate the production of singlet oxygen [20]. Then we inserted the tip of the PDT device inside the cell as shown in the images displayed in Fig. 2c, e and the magnified images shown in Fig. 1f, g. It is observed that the tumor cell is a rapidly dividing cell, with destroyed outer barriers as shown in the image of Fig. 2f. These are the basic properties of tumor cell along with other such as altered enzymes and increased blood perfusion [26]. Then the cancer cell is exposed to UV environment for 10 min while the PDT device is inserted inside the cell. This is shown in the case displayed by the image in Fig. 2g. In Fig. 2g, the tip has been prepared as mentioned above with ZnO NRs conjugated with protoporphyrin. The basic needs for PDT is the availability of the molecular oxygen, through a photosensitizer excited by white light, which in our case can be emitted by ZnO nanorods [20, 21]. Then there is an energy exchange process between the conjugated tip and the molecular oxygen inside the cell. The phototoxic reaction involves the formation of singlet oxygen in the cell which causes initial damage to mitochondria leading to cell necrosis as shown in image of Fig. 2h [22]. The image shown in Fig. 2i displays only tip of the PDT device after cell necrosis. The tip appears thicker after the manipulation inside the cell because some remnants' of the cell are attached to it. We used a light exposure time of 10 min in many similar different repetition experiments and found similar result. It is noted that the tip of the present PDT device can be used only once for these local single cell experiment. The reason is that all the remnants of the cell i.e. calcium, protein, etc., which are parts of the membrane, stick on the tip and they will decrease the intensity of light used for cell necrosis. The control experiment using a bare ZnO NRs tip (without the PPDME) was also performed. Similar UV excitation light exposure time of 10 min was used. No immediate cell necrosis was observed as expected due to the absence of the PPDME.

Figure 3 shows a schematic diagram of the PDT mechanism involved in the present study. The mechanism starts with the excitation of the photosensitizer inside the cancer cell and the release of active species of oxygen which then leads to cellular toxicity and finally cell necrosis. ZnO NRs are excited by the UV and consequently emits white light which is absorbed by the PPDME (630 nm) [19]. In turn, singlet oxygen is released to kill the mitochondria causing finally the required cell necrosis.

Fluorescence spectrum (Fl) of both the bare and the conjugated PDT devices was also measured to identify the emission nature. Figure 4a shows the Fl of the bare ZnO NRs-based PDT device measured at room temperature. This Fl spectrum shows five different peaks. These are the UV and the rest belong to the white light constituents. The first dominant Fl peak was observed at 337 nm and the origin of this peak is to further be investigated. The second peak at 395 nm is attributed to the recombination of free excitons in ZnO i.e. band edge emission [27]. The third blue emission peak at 468 nm comes from the combination of zinc interstitial level to valence band combined with

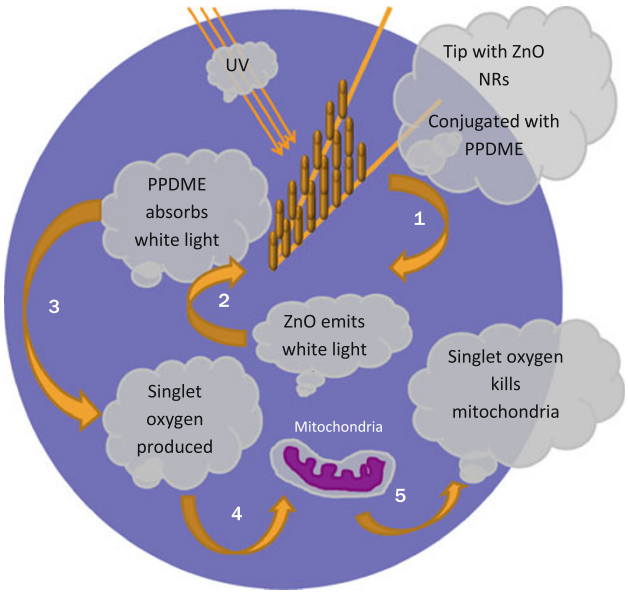


**Fig. 3** Schematic diagram showing the intracellular PDT process of cancer cell

emission due to transmission from the zinc interstitial to zinc vacancies in ZnO [28]. The fourth green emission 560 nm is attributed to a recombination of electrons at the conduction band with holes trapped in oxygen- and zinc-related defects [29]. The fifth peak is at 680 nm is attributed to other deep level defect-related radiative transition in ZnO [28]. The stronger intensity of the blue emission relative to the UV emission can suggest the non-stochiometric composition of ZnO [30]. Figure 4b shows the fluorescence emission of PDT-conjugated ZnO NRs device. Basically, it is consisting of the same peaks, except a small change of the nature probably due to the absorption by the PPDME. Moreover, the intensity of the Fl in Fig. 4b is five times less than that of the Fl spectrum in Fig. 4a indicating absorption of some of the emitted white light by the PPDME as required. From the combined results shown in Figs. 2 and 4, we concluded that white light emission from ZnO was transferred by absorption to the PPDME, which in turn caused excitation and production of singlet oxygen which consequently caused the aimed cell necrosis.

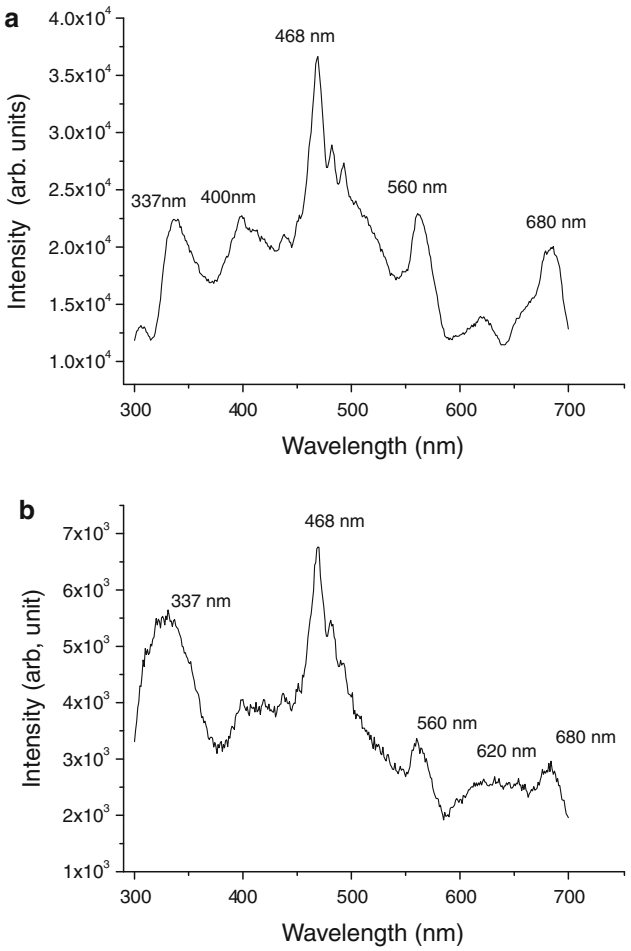


**Fig. 4** Florescence (Fl) spectra of ZnO NRs, without (**a**) and (**b**) with protoporphyrin, respectively. These spectra were taken using a laser line of wavelength of 240 nm as an excitation source from a PTI-Fluorescence system

## Conclusion

ZnO nanorods being an intrinsic white light–emitting material have been demonstrated as an efficient light system attached to a photosensitizer for intracellular cell necrosis. ZnO nanorods grown on the femto tip are shown to deliver the photosensitizer to breast cancerous cells and causes the necrosis within few minutes. Topical pain caused by the conventional PDT method can be reduced by this technique. Exceptional care must be taken to avoid the PDT device tip from entering into the lymphatic vessels of the breast to minimize the chance of spread of cancer cells. We present integrated, affordable and improved photosensitizer delivery system which can be used for local cancer cell necrosis. This device opens an era for effective drug delivery for specific localized tumors with reduced pain. We have developed another research tool in which, instead of using many nanorods on the tip, we can produce singlet oxygen in the cell, using single ZnO nanorod with some nanometers accuracy. This will be published in our next paper.

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