Video Article

Photodynamic Therapy with Blended Conducting Polymer/Fullerene Nanoparticle Photosensitizers

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Abstract

In this article a method for the fabrication and reproducible *in-vitro* evaluation of conducting polymer nanoparticles blended with fullerene as the next generation photosensitizers for Photodynamic Therapy (PDT) is reported. The nanoparticles are formed by hydrophobic interaction of the semiconducting polymer MEH-PPV (poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene]) with the fullerene PCBM (phenyl-C_{61}-butyric acid methyl ester) in the presence of a non-compatible solvent. MEH-PPV has a high extinction coefficient that leads to high rates of triplet formation, and efficient charge and energy transfer to the fullerene PCBM. The latter processes enhance the efficiency of the PDT system through fullerene assisted triplet and radical formation, and ultrafast deactivation of MEH-PPV excited stated. The results reported here show that this nanoparticle PDT sensitizing system is highly effective and shows unexpected specificity to cancer cell lines.

Video Link

The video component of this article can be found at https://www.jove.com/video/53038/

Introduction

In Photodynamic Therapy (PDT) photosensitizers are administered to target tissue, and upon exposure to light the photosensitizer generates Reactive Oxygen Species (ROS). ROS species such as singlet oxygen and superoxide can induce oxidative stress and subsequent structural damage to cells and tissue[1-4]. Due to its ease of application this method has been actively investigated and clinical trials have taken place[5,6]. However, significant issues such as dark toxicity of the sensitizers, patient sensitivity to light (due to non-selective distribution of the sensitizer), and hydrophobicity of the sensitizers (which leads to reduced bioavailability and potential acute toxicity) remain.

Here we report a method for the fabrication and *in-vitro* evaluation of conducting polymer nanoparticles blended with fullerene as the next generation photosensitizers for PDT. The nanoparticles are formed by self-aggregation of the semiconducting polymer MEH-PPV (poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene]) with the fullerene PCBM (phenyl-C_{61}-butyric acid methyl ester) when these materials dissolved in a compatible solvent are rapidly injected into a non-compatible solvent (**Figure 1A**). The choice of MEH-PPV as the host polymer is motivated by its high extinction coefficient that leads to high rates of triplet formation, and both efficient and ultrafast charge and energy transfer to the fullerene PCBM[7]. These properties are ideal for sensitization of singlet oxygen and superoxide formation in PDT.

Fullerene has in fact been applied in PDT in both molecular and nanoparticle form[8-13]. However, severe cytotoxicity has hampered further development[12]. Here we show that encapsulating the fullerene in a host matrix of MEH-PPV to yield composite MEH-PPV/PCBM nanoparticles results in a PDT sensitizing material that is not intrinsically cytotoxic, shows specificity towards cancer cells due to nanoparticle size and surface charge, and yields highly effective PDT treatment at low light doses due to the aforementioned photophysical properties.

Protocol

1. Culturing Cell Lines

1. Thaw TE 71 (Mouse thymic epithelial cells), MDA-MB-231 (Human breast cancer cells), A549 (Human lung cancer cells) and OVCAR3 (Human ovarian tumor cells) by holding the cryogen vials in warm water for less than 2 min. Add 10 ml DMEM media supplemented with 10% FBS to each cell line and centrifuge for 6 min at 106 x g.

2. Aspirate the suspension and add 3ml media to the pellet. Mix the cells properly by pipetting several times. Add this cell solution to pre-warmed 7 ml DMEM media supplemented with 10% FBS in T75 flasks and keep the flasks in humidified atmosphere of 95% air/ 5% CO_2 at 37 °C. Label this flask as Passage 0.
3. When the confluency of the cells reaches 80%, harvest the cells by incubating them with 0.05% trypsin for 10 min. Neutralize the trypsin by adding equal amount of media. Centrifuge this solution for 6 min at 106 x g. Remove the suspension and add 3 ml fresh media to it. Mix well and transfer small amount (100 µl) to a culture flask containing 7 ml media. Incubate the culture flask in incubator. Label the flask as Passage 1.
4. Culture the cell lines until passage 11 or 12.

2. Fabrication of Nanoparticles

1. Preparation of ~10^{-6} M (adjusted) undiluted MEH-PPV stock solution
    1. In a vial add 1mg Poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV) with molecular weight (average Mn) 150,000-250,000 g/mol and 3 ml tetrahydrofuran (THF). Stir the mixture for 2 hr while heating at 80 °C on a hot plate.
    2. Filter the above solution in a new vial using a 0.2 µm syringe filter. Label this solution as 'undiluted MEH-PPV stock solution'. This solution will be involved in nanoparticle preparation after fine tuning of the concentration as described in steps 2.1.3 and 2.1.4.
    3. Add 50 µl of the undiluted MEH-PPV stock solution into 3 ml THF. Label this solution as 'diluted MEH-PPV stock solution'. Transfer to a 1 cm quartz cuvette and measure the absorbance at 495 nm by UV-vis spectroscopy.
    4. If the absorbance of the diluted MEH-PPV stock solution is higher than 0.17, dilute the undiluted MEH-PPV stock solution by adding more THF 1 ml at a time, and repeat step 2.1.3 until the measured absorbance at 495 nm is in the range 0.13-0.17.
    5. Calculate the molarity of the diluted MEH-PPV stock solution by using Lambert-Beer law as shown below. Here 0.15 absorbance is used as an example for the remainder of the protocol (*i.e.*, adjust all following calculations based on observed absorbance), the MEH-PPV extinction coefficient used is 10^7 M^{-1} cm^{-1} and the path length used is 1 cm.
    $A = \varepsilon \times b \times c$ (Eqn 1)
    (ε - the molar extinction coefficient, A – absorbance, b – path length, c – concentration of the solution)
    $0.15 = 10^7$ M^{-1} cm^{-1} x 1 cm x c (Eqn 2)
    c = 1.5 x 10^{-8} M (Eqn 3)
    Use this concentration to find the concentration of the undiluted MEH-PPV stock solution as follows:
    $M_1V_1 = M_2V_2$ (Eqn 4)
    0.15 x 10^{-7} M x 3050 µl = M_2 x 50 µl (Eqn 5)
    M_2 = 9.15 x 10^{-7} M (Eqn 6)
    This is the concentration of the 'adjusted undiluted MEH-PPV stock solution'.

2. Calculating mass of MEH-PPV in the adjusted undiluted MEH-PPV stock solution
    1. Calculate the mass of MEH-PPV in 1 ml of the adjusted undiluted stock solution as shown below using the molarity obtained in section 2.1.4 and the molecular weight (M_w) of MEH-PPV, which is 10^6 g/mol.
    M = n/V (n – no. of moles, V – volume in L) (Eqn 7)
    Thus, the mass of MEH-PPV in 1 ml of adjusted undiluted stock solution is 9.15 x 10^{-4} g.

3. Blending PCBM in MEH-PPV
    1. Calculate the mass of Phenyl-C_{61}-butyric acid methyl ester (PCBM) to be added into the stock MEH-PPV solution to make 50 wt% PCBM doped MEH-PPV solution, where 50 wt% PCBM is defined with respect to the mass of MEH-PPV (*i.e.,* half the mass of MEH-PPV). By using the weight of MEH-PPV obtained in section 2.2.
    Mass of PCBM/9.15 x 10^{-4} g of MEH-PPV x 100% = 50 wt% PCBM (Eqn 8)
    Mass of PCBM = 4.57 x 10^{-4} g (Eqn 9)
    2. Weigh 1 mg PCBM in a vial and add 500 µl THF. Calculate the concentration of PCBM in this solution using the molecular weight of PCBM as 910.88 g/mol
    Molarity of PCBM solution = 0.001 g/(910.88 g/mol * 500 µl)(Eqn 10)
    Molarity of PCBM solution = 2.19 x 10^{-9} mol/µl(Eqn 11)
    3. Calculate the volume of PCBM solution needed to add to the adjusted undiluted MEH-PPV stock solution to obtain the 50 wt% PCBM doped MEH-PPV solution in THF by using the molarity calculated in step 2.3.2
    4.57 x 10^{-4} g of PCBM x 1 mol/910.88 g x 1 µl/2.19 x 10^{-9} mol = 229 µl (Eqn 12)
    Add 229 µl of the PCBM solution into 1 ml of adjusted undiluted MEH-PPV stock solution and mix well.

4. Preparation of nanoparticles by reprecipitation method
    1. Transfer 1 ml of the blended MEH-PPV/PCBM solution into the 1 ml syringe with the needle attached to it.
    2. Rapidly inject 1 ml of the blended MEH-PPV/PCBM solution into 4 ml of DI water stirring at 1,200 rpm. Stop stirring immediately after injection. Use these nanoparticles without further processing.

3. Incubation of Cell Lines with Nanoparticles for Imaging

NOTE: All the imaging experiments were completed in 35 mm petri dishes

1. Uptake of nanoparticles in cell lines
    1. Culture cell lines up to the 12^{th} passage. At the 12^{th} passage culture cells in 35 mm Petri dishes. Adjust concentration of cells added to the 35 mm Petri dishes such that after 24 hr the cells are 40% confluent.
    2. At this stage remove the DMEM media supplemented with 10% FBS from the petri dishes, wash the cells with 1x DPBS twice, and add 100 µl of the nanoparticles suspension into 2 ml DMEM to the Petri dishes.

3. After 24 hr remove the DMEM/nanoparticles suspension from the petri dishes and wash the cells with 1x DPBS 3 times. Then fix the cells by incubating with 4% paraformaldehyde for 10 min. Wash twice with DPBS. Stain the cells with 300 nM DAPI by incubating with the dye for 2 min. Wash twice with DPBS. Then keep the cells in DPBS for imaging.

2. Detection of ROS
    1. Culture A549 and OVCAR3 cell lines in Petri dishes, 6 per cell line, as explained in section 3.1.1. Label the Petri dishes as shown in the **Table 1**.
    2. Add 100 µl of the nanoparticle suspension into 2 ml DMEM to three of the petri dishes as shown in the **Table 1** and incubate for 24 hr. For the Petri dishes that will receive light doses, wash the cells after 24 hr and suspend the cells in HBSS (Hank's Balanced Salt Solution) dye free media.
    3. Warm up the lamp of the solar simulator for 15 min. Place UV filter in front of the lamp to filter out UV light. Calibrate the lamp with a reference solar cell by adjusting the lamp power to obtain 0.5 sun (50 mW/cm^2) intensity at the surface of the petri dish. In this particular setup that condition was achieved with 218 W power supplied to the lamp.
    4. Place the petri dishes under the lamp (lid open) for 60 min, which results in a light dosage of 180 J/cm^2 as shown in the calculations below:
    $50\ mW/cm^2 \times 3{,}600\ sec/1{,}000 = 180\ J/cm^2$
    5. Remove HBSS and without washing further add 2 ml DMEM media supplemented with 10% FBS to the petri dishes. Incubate the cells for another 2 hr.
    6. For the positive control incubate the cells with 100 µM hydrogen peroxide (H_2O_2) for 30 min.
    7. Stain the cells in all the petri dishes with a final concentration of 5 µM of the ROS detecting reagent by incubating the cells with the dye for 30 min at 37 °C.
    8. Fix the cells by incubating with 4% paraformaldehyde for 10 min. Wash twice with DPBS. Stain the cells with 300 nM DAPI by incubating with the dye for 2 min. Wash twice with DPBS. Then keep the cells in DPBS for imaging.

3. Apoptosis and necrosis by PI and annexin V FITC
    1. Culture each cell line in 5 Petri dishes. Three of these Petri dishes will be for experiment while the remaining 2 petri dishes will be control samples. Incubate the 3 Petri dishes for experiment with nanoparticles as explained in section 3.1.2. The control samples are not incubated with nanoparticles.
    2. After 24 hr incubation follow the step 3.2.3.
    3. Place the 3 experimental petri dishes under the lamp (lid open) and remove one at 20 min, one at 40 min and one at 60 min. This yields three samples that were exposed to light doses of 60, 120 and 180 J/cm^2, respectively (calculation in section 3.2.4). Next, administer a 180 J/cm^2 light dosage to one of the control petri dishes. This is the control with light dose applied in the absence of nanoparticles. Do not apply light dose to the remaining control sample (no nanoparticles and no light dose applied).
    4. Replace the HBSS with DMEM media supplemented with 10% FBS and keep the petri dishes in the incubator for 4 hr.
    5. Stain the cells in the experimental and the control petri dishes with 20 µl of annexin V FITC by incubating the cells with the dye for 15 min. Wash twice with DPBS. Stain the cells with 300 nM DAPI as well as 300 nM PI (propidium iodide) by incubating with the dyes for 2 min. Wash twice with DPBS. Then keep the cells in DPBS for imaging.

4. Intrinsic Cytotoxicity of Nanoparticles

1. Counting cells and culturing in 96-well plates
    1. Harvest the cells from culture flasks by removing media and washing the cells twice with DPBS followed by incubation of the cells with 0.05% trypsin for 10 min. Add 2 ml DMEM media supplemented with 10% FBS to the resulting cell solution. Mix the solution properly to separate cell clusters into singlets.
    2. Take 100 µl of the cell suspension and add to 900 µl DMEM media supplemented with 10% FBS. Mix well. Place 10 µl of this suspension onto a hemocytometer. Count the cells using the hemocytometer and adjust the concentration of the cell suspension in 4.1.1 to 5×10^4 cells/ml.
    3. Take 5 96-well plates labeled as 0 hr, 24 hr, 48 hr, 72 hr and 96 hr. Add 50 µl of the 5×10^4 cells/ml cell solutions (TE 71 and A549) into the wells as shown in **Table 2**, thus seeding 2,500 cells/well. Do the same procedure for OVCAR3 and MDA-MB-231 cell lines.
    4. After 24 hr wash the wells with 1x DPBS and add 50 µl of increasing concentrations of nanoparticles into the wells as shown in **Table 2**. Prepare different nanoparticle concentrations by adding 20, 100, and 180 µl of nanoparticle suspension from 2.4.2 to DMEM to obtain a final volume of 2 ml, which yields nanoparticle concentrations of 0.4×10^{-4} mg/ml, 2.0×10^{-4} mg/ml, and 3.6×10^{-4} mg/ml, respectively. Each cell line has triplicates for each concentration of nanoparticles.

2. Measuring the cell viability in dark
    1. Add 10 µl MTT to the 0 hr plate immediately after adding nanoparticles. Incubate the plate for 4 hr for formazan crystals to form. Add 50 µl solubilization solution into the wells. Incubate the plate for 6 hr to dissolve the formazan crystals.
    2. Measure the cell viability of the 0 hr plate by recording the absorbance at 570 nm with microplate reader.
    3. For 24 hr plate, wash the cells with 1x DPBS and add 50 µl media in each well after 24 hr incubation with nanoparticles. Add MTT and read the plate as explained in 4.2.1 and 4.2.2.
    4. For 48 hr, 72 hr, and 96 hr plates, wash the cells with 1x DPBS and add 50 µl media into the wells after 24 hr incubation with nanoparticles. Incubate the cells for the remaining time periods.
    5. 4.2.5) Measure the cell viability at the particular time points as explained in 4.2.1 and 4.2.2.
    6. Repeat the complete experiment for 3 times (n = 3)

5. Measuring Cell Viability After PDT

1. Counting cells and culturing in 96-well plates.
    1. Label a set of 96-well plates as shown in **Table 3**, both for the TE 71 + A549 plates and OVCAR3 + MDA-MB-231 plates. The layout of the plates will be the same as shown in 4.1.3.
    2. Seed the 96-well plates as explained in section 4.1 with the same layout as shown in **Table 2** in section 4.1.2.
    3. After 24 hr add nanoparticles to the plates as explained in 4.1.4.
    4. 24 hr after addition of nanoparticles wash the cells with 1x DPBS and add 50 µl HBSS to each well.
    5. Irradiate the plates with the respective light doses as explained in 3.2.3.
    6. Replace the HBSS with 50 µl DMEM media supplemented with 10% FBS and incubate the plates for the respective time periods after PDT.
    7. After each time period measure the cell viability as explained in 4.2.1 and 4.2.2.

6. Fluorescence Microscopy

1. Uptake of nanoparticles
    1. Turn on the lamps of the microscope and the laser 30 min before imaging. Put the petri dish containing the fixed cells (as explained in section 3.1) on the stage of the microscope.
    2. Collect the fluorescence from nanoparticles and DAPI by using filters as shown in **Table 4**. Overlay the phase contrast, nanoparticle and DAPI images in ImageJ software.

2. Detection of ROS
    1. Turn on the lamps of the microscope 30 min before imaging. Put the petri dish containing the cells (as explained in section 3.2) on the stage of the microscope.
    2. Collect the fluorescence from nanoparticles, DAPI, and the ROS detecting reagent by using filters as shown in **Table 4**. Overlay the phase contrast, nanoparticle, DAPI and ROS detecting reagent images in ImageJ software.

3. Apoptosis and necrosis by PI and annexin V FITC
    1. Turn on the lamps of the microscope 30 min before imaging. Put the Petri dish containing the cells (as explained in section 3.3) on the stage of the microscope.
    2. Collect the fluorescence from nanoparticles, DAPI, PI, and Annexin V FITC by using filters as shown in **Table 4**. Overlay the phase contrast, nanoparticle, DAPI, PI and Annexin V FITC images in ImageJ software.

Representative Results

Uptake and intrinsic cytotoxicity of nanoparticles

The 50 wt% blended MEH-PPV/PCBM nanoparticles were incubated with TE 71, MDA-MB-231, A549 and OVCAR3 cell lines. The PCBM blending level was chosen as 50 wt% PCBM, which has been shown to provide ideal charge and energy transfer properties between conjugated polymers and fullerenes[14]. Fluorescence images of nanoparticle uptake are shown in **Figure 1B**. Cells were incubated for 24 hr with nanoparticles to ensure nanoparticle uptake. Cells were then fixed with 4% paraformaldehyde before imaging, and stained with DAPI in order to detect cells and the location of the nucleus. In order to image sufficiently separated cells 40% confluency was maintained. Fluorescence images with corresponding phase contrast images show that there is preferential uptake of nanoparticles by the A549 and OVCAR3 cancer cell lines. No detectable fluorescence can be seen in TE 71 control and MDA-MB-231 cancer cell lines, indicating limited uptake. On the other hand, A549 and OVCAR3 cancer cell lines exhibit significant nanoparticle uptake. Intrinsic cytotoxicity (dark toxicity) was evaluated by incubation of the 50 wt% blended MEH-PPV/PCBM nanoparticles with TE 71, MDA-MB-231, A549 and OVCAR3 cell lines and quantifying the cell viability by MTT assay. MTT data in **Figure 1C** show normal proliferation of the cell lines.

Nanoparticles as the source of ROS

To ensure that the nanoparticles are the source of ROS, and only after exposure to light, ROS formation was evaluated with an ROS detecting reagent kit. Data for OVCAR3 are shown in **Figure 2**. Absence of green emission in **Figure 2A**-**C** indicates ROS are not formed for the control samples. Bright green emission from the ROS detecting reagent is observed for samples treated with nanoparticles and exposed to light as shown in **Figures 2D** and **E** (immediately after PDT and 2 hr after PDT), confirming that ROS are generated during PDT.

PDT

The performance of MEH-PPV/PCBM nanoparticles in PDT was quantified by MTT assay immediately after PDT, and after 4 and 12 hr post-incubation periods. The data are shown in **Figure 3** for the 4 hr post-incubation period. The A549 and OVCAR3 cancer cell lines exhibit significant cell death after PDT treatment: up to 60% for A549 and 100% for OVCAR-3. The TE 71 control and MDA-MB-231 cancer cell lines show limited effects. TE71 is a normal control cell line and is not expected to internalize nanoparticles. Only low non-specific uptake of nanoparticles by TE-71 is observed experimentally. Low nanoparticle uptake is also observed for MDA-MB-231, which is in this case due to the lower metabolic rate compared to the other cancer cell lines. The PDT data show that the MEH-PPV/PCBM nanoparticles are highly effective PDT sensitizers, and that the PDT effectiveness scales with nanoparticle uptake. The differences in PDT results between the cancer cell lines considered here are due to the difference in aggressiveness (metabolism and rate of endocytosis) between these cell lines.

Progression of PDT-induced cell death

Live/dead double staining with PI and Annexin V FITC provides information on necrotic and apoptotic mechanisms of cell death. This staining scheme was applied to the cell lines studied here after PDT to learn more about PDT-induced cell death pathways. **Figure 4** shows epiluminescence images of TE 71and OVCAR3 cell lines stained with Annexin V FITC, PI and DAPI. The data show that there is no effect of PDT on the TE 71 control cell line, consistent with the negligible uptake of nanoparticles. The same observation was made for MDA-MB-231 (data not shown). When OVCAR3 underwent PDT at 60 and 120 J/cm^2 dual staining of PI (purple) and Annexin V FITC (green) was observed. At 180 J/cm^2 only the PI stain was observed, suggesting acute necrotic cell death under that condition.

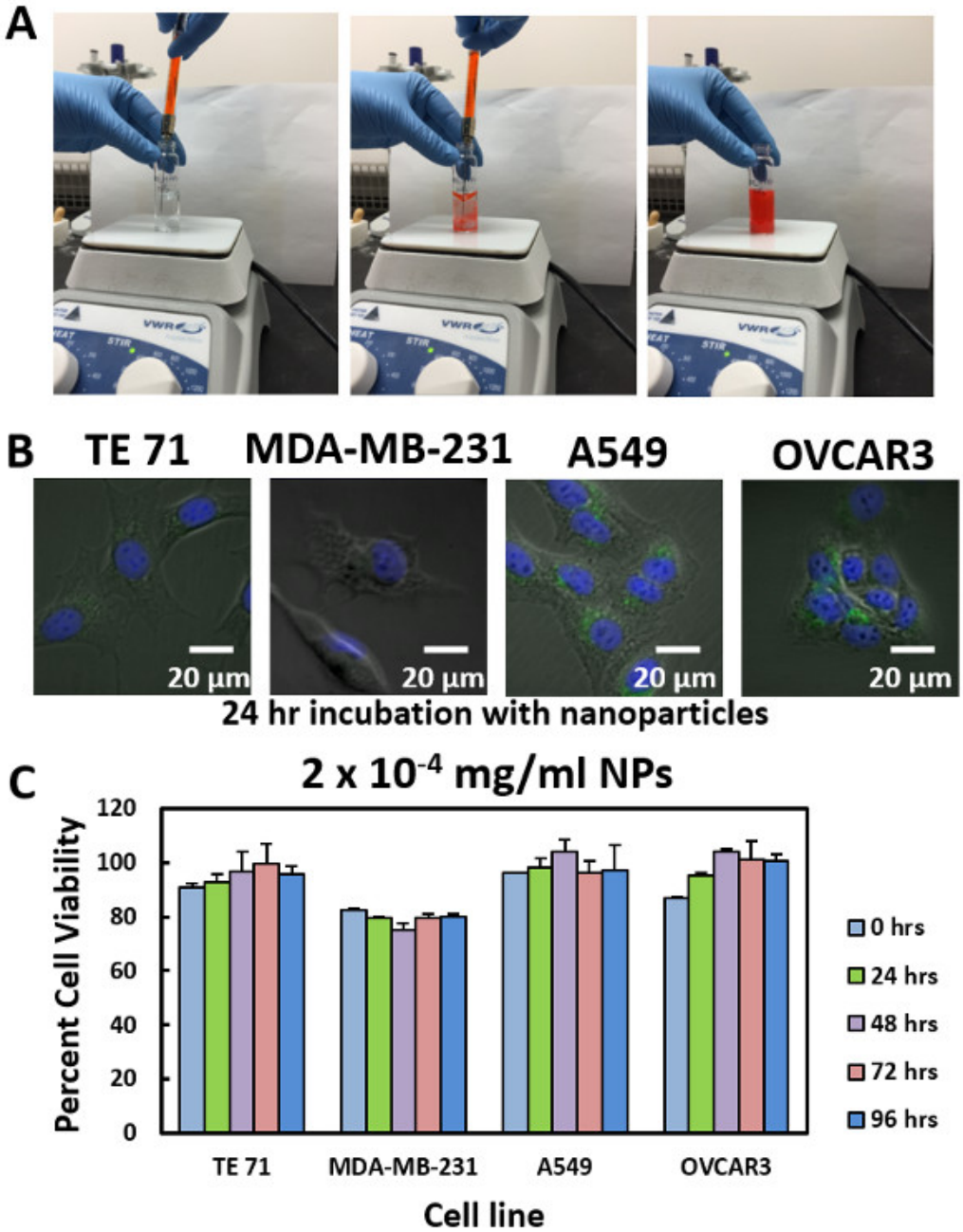

Figure 1. (**A**) Fabrication of nanoparticles by reprecipitation method, (**B**) TE 71, MDA-MB-231, A549 and OVCAR3 cell lines incubated with nanoparticles. The nanoparticles are shown in green color, the nucleus is shown in blue color. The fluorescence images are overlaid with the phase contrast images, scale bar = 20 µm, (**C**) Intrinsic cytotoxicity of nanoparticles evaluated by measuring the cell viability for each cell line up to 96 hr. The cell viabilities are compared with control dose of nanoparticles (0 mg/ml) by setting the viability of control samples at 100% (not shown). Error bars are the standard deviations for results from 3 separate experiments (n = 3). Please click here to view a larger version of this figure.

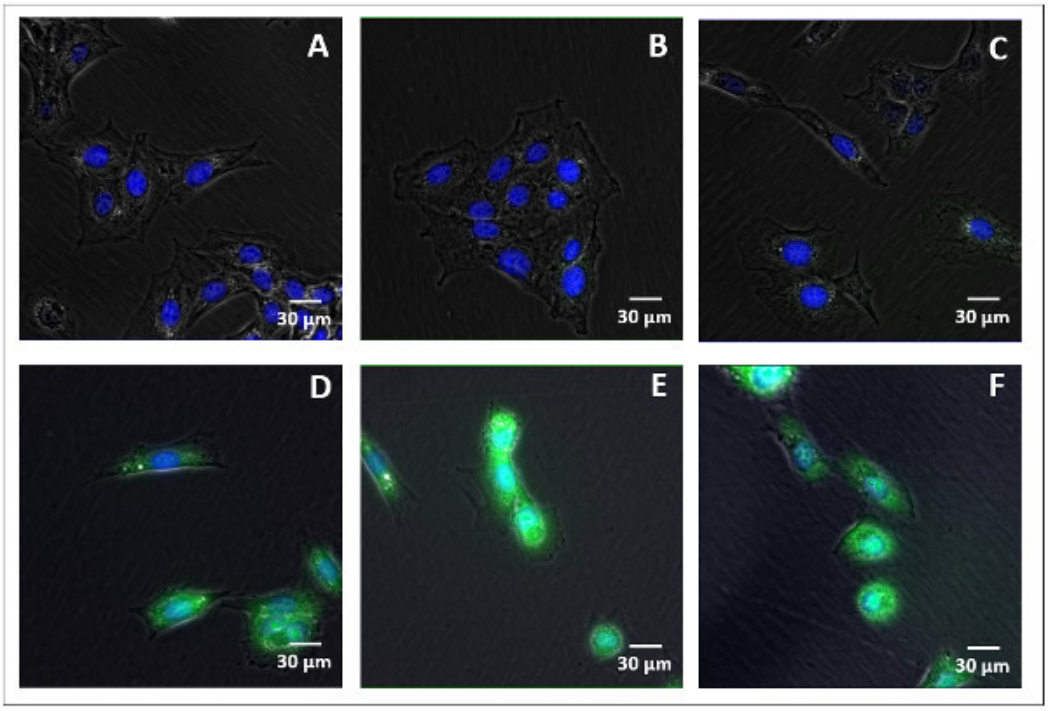

Figure 2. Detection of ROS in OVCAR3 cell line with ROS detecting reagent. (**A**) no nanoparticles, no light exposure, (**B**) no nanoparticles, exposed to 180 J/cm^2 light, (**C**) 2 x 10^{-4} mg/ml nanoparticles, no light exposure, (**D**) with nanoparticles and exposure to 180 J/cm^2 light, taken immediately after treatment, (**E**) 2 hr post-PDT, (**F**) with 100 µl H_2O_2 as positive control. The bright green emission in D-F is from the ROS detecting reagent confirming ROS formation. Scale bar = 30 µm. Please click here to view a larger version of this figure.

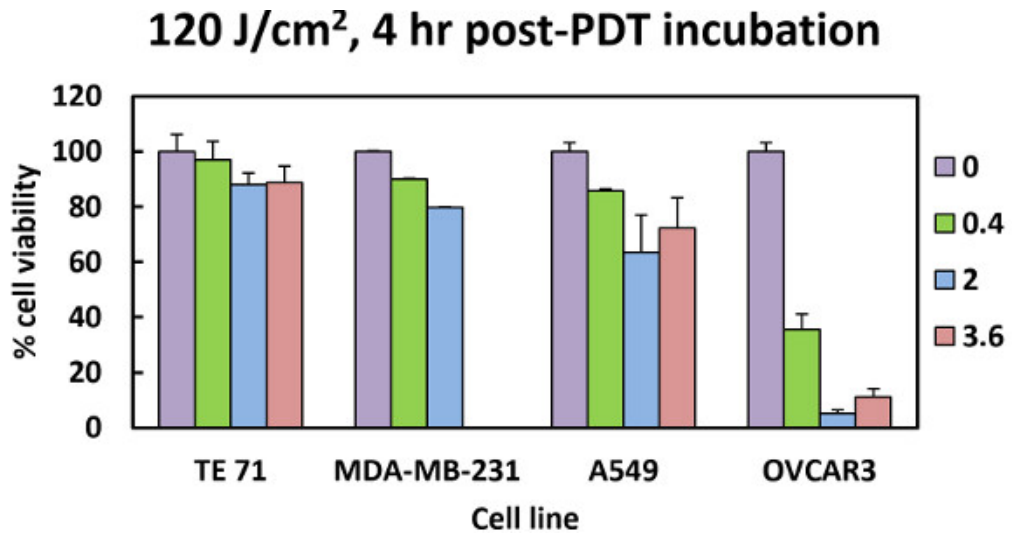

Figure 3. Cell viabilities of TE 71, MDA-MB-231, A549, and OVCAR3 cell lines administered with increasing doses of nanoparticles and irradiated with 120 J/cm^2 light dose. The post-PDT incubation time is 4 hr. The nanoparticle doses in the legend are in 10^{-4} mg/ml. Error bars are the standard deviations for results from 3 separate experiments (n = 3). Please click here to view a larger version of this figure.

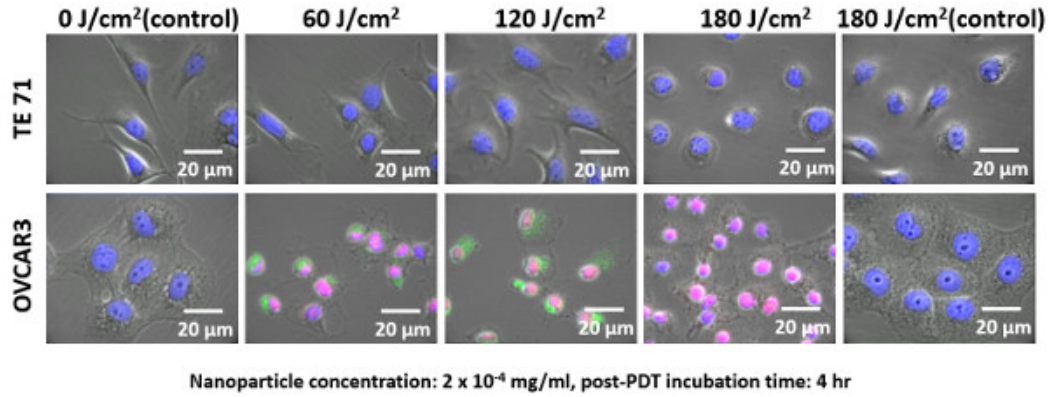

Figure 4. Live/dead staining of TE 71 and OVCAR3 cell lines with annexin V FITC and PI. Green emission corresponds to annexin V FITC, purple emission corresponds to PI (red, mixed with blue DAPI emission), and blue emission corresponds to DAPI nuclear stain. Images are the overlay of phase contrast and epiluminescence images. Scale bar = 20 µm. Please click here to view a larger version of this figure.

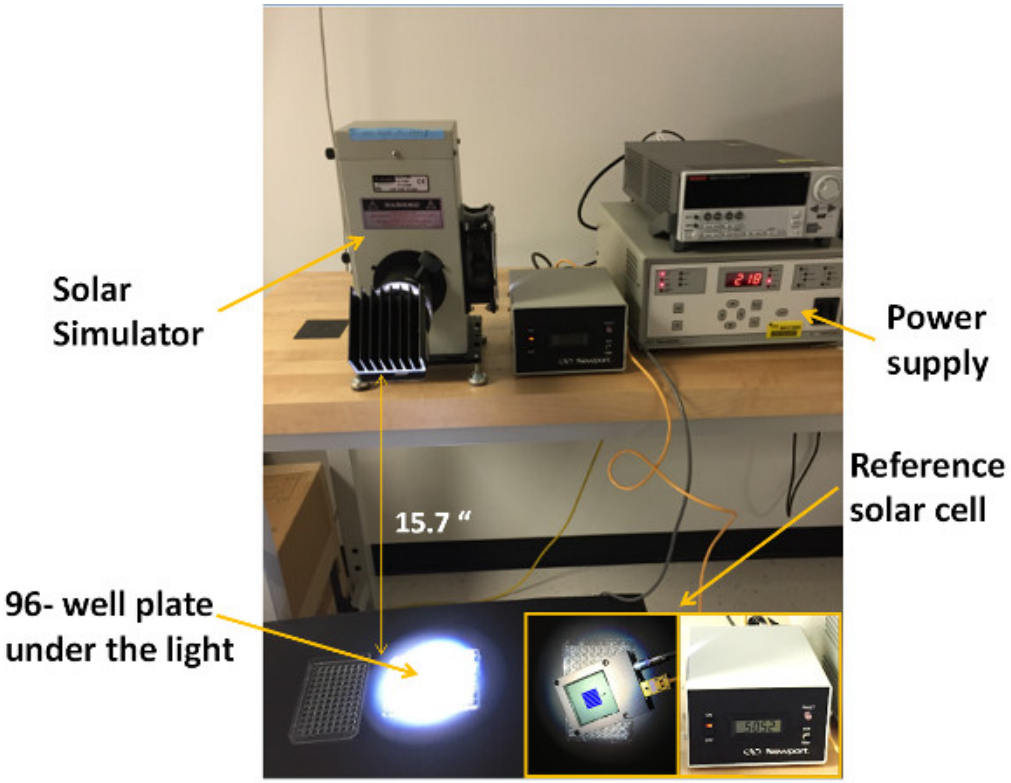

Figure 5. Solar simulator setup for exposure of cells with calibrated light. A reference solar cell was used for calibration, shown in the inset registering 0.5 sun light intensity (50 mW/cm^2). Please click here to view a larger version of this figure.

Negative Controls	No NPs, no light	No NPs, with light	No light, with NPs
Positive control	100 µl H_2O_2 (No NPs, no light)	---	---
Experimental	0 hr post PDT (NPs and light)	2 hr post PDT (NPs and light)	---

Table 1: Experimental design for detection of ROS.

0 hr plate	Media			TE 71			A549			Media		
No treatment												
0 mg/ml												
0.4 x 10^{-4} mg/ml												

2.0×10^{-4} mg/ml												
3.6×10^{-4} mg/ml												

Table 2: Layout of the 96-well plate for incubation of nanoparticles to evaluate intrinsic cytotoxicity of nanoparticles/PDT effect

Plate no.	Light dose (J/cm^2)	Post PDT time (hr)
1	60	0
2	60	4
3	60	12
4	120	0
5	120	4
6	120	12
7	180	0
8	180	4
9	180	12

Table 3: Labeling the 96-well plates for PDT evaluation.

Fluorophore	Excitation filter	Dichroic mirror	Emission filter	Microscopy	Excitation source
Nanoparticle	488/10	500 LP	510 LP	Confocal	Ar-Kr ion laser
DAPI	350/52	405 LP	450/20	Epiluminescence	Mercury lamp
PI	543/22	562	592/40	Epiluminescence	Mercury lamp
Annexin V FITC	483/30	505 LP	535/40	Epiluminescence	Mercury lamp
ROS detecting reagent	491/10	510 DCLP	525/50	Epiluminescence	Mercury lamp

Table 4. Microscopy configuration for imaging experiments.

Discussion

To achieve nanoparticle uptake it was necessary to maintain some critical measures while fabricating the nanoparticles. A 10^{-6} M MEH-PPV solution (blended with 50 wt% PCBM) in THF was prepared to inject into DI water, as it was observed that the concentration of this solution plays an important role in determining the size of nanoparticles being formed. Concentration was checked by UV-vis spectroscopy. Note that in protocol step 2.1.3 it was necessary to dilute the initially prepared MEH-PPV solution (undiluted MEH-PPV stock solution) first before taking UV-vis spectra since this solution has an absorbance much greater than 1. The speed of injection also plays a critical role in deciding the size of the nanoparticles, and has to be as fast as possible while vigorously stirring the DI water. Slow injection will result in bigger nanoparticles. Also, the stirring should be stopped immediately after injection to avoid further aggregation. While injecting the solution in water it is necessary to keep the needle near the inner surface of the vial while inserting the needle completely into the solution to avoid bubble formation, which will affect the size of the nanoparticles. In our experiments nanoparticle sizes obtained were 61.5 ± 23.3 nm as measured by DLS. DLS was chosen instead of TEM as it is fast, inexpensive, reliable for this size and easily available. The zeta potential on these nanoparticles was found to be -9.66 ± 8.12 mV, *i.e.,* slightly negative to neutral surface charge.

It was essential to count the cells while evaluating the intrinsic cytotoxicity of nanoparticles and quantify PDT results, as these techniques are based on the MTT assay which provides a quantitative measurement of cell viability. It is essential to start the experiment with the same number of cells in each well of the 96-well plate, which allows for comparison of cell viability with respect to the dose of nanoparticles and light as well as the control experiments.

A solar simulator was used to irradiate the samples. The setup is shown in **Figure 5**, and consists of the light source, a UV filter, and a reference solar cell. With this illumination scheme a high degree of control of the spectral properties and intensity of the light source could be achieved, which resulted in highly reproducible results. It is very important to realize that most light sources do not provide a uniform intensity profile. The solar simulator, however, can be aligned to accomplish near uniform intensity in the area of illumination. This was verified by a reference solar cell in different regions of the illuminated area. We also took care to always place plates in the same region of the light spot and in the same orientation to further minimize effects of variation in intensity. The light source to sample distance indicated in **Figure 5** provided us with 50 mW/cm^2 (0.5 sun) of intensity under an electrical lamp power of 218 W. For these experiments HBSS dye free media was used as to avoid absorption of light by indicator dyes. After PDT, the cells were again incubated for certain time periods at 37 °C (post-PDT incubation) to observe the progress of PDT.

Staining of cells required some trial and error to find the correct concentration of the respective dye. This is achieved by repeating the experiment while increasing the dye concentration steadily until appropriate results were achieved.

The method also has a couple of limitations, specifically regarding the nanoparticle system and PDT treatment. Since nanoparticles are prepared by the reprecipitation method there is some variability in the obtained nanoparticle size from batch to batch, and some polydispersity in nanoparticle size exists that cannot be controlled. It has also not been possible to make nanoparticles less than 20 nm in size. These limitations could be challenges in developing small size monodisperse nanoparticles that can be applied *in vivo*. Furthermore, the *in-vitro* PDT experiment requires the cells to be outside the incubator for an extended amount of time, which could impose some stress on the cells.

The method discussed herein for PDT is significant with respect to current approaches. PDT using small molecule sensitizers and sensitizer doped nanoparticles has seen limited clinical application due to significant issues with dark toxicity of the sensitizers, patient sensitivity to light (due to non-selective distribution of the sensitizer), and hydrophobicity of the sensitizers (which leads to reduced bioavailability and potential acute toxicity). In surgery, even if the tumor is removed from the body, a few cancer cells remain and can result in remission. In radiotherapy and chemotherapy normal tissue is affected also.

In summary, we have shown that a next generation photosensitizer based on conducting polymer nanoparticles is a promising design for PDT applications due to the absence of dark toxicity, effective ROS generation, reasonable selectivity of uptake, and ability to induce abundant cell death. In the near future these nanoparticles will be further modified for targeting of cell surface receptors in order to achieve enhanced uptake and selectivity. Conducting polymers containing heavy atoms or metal centers will be considered as well for their enhanced intersystem crossing rates.

Disclosures

The authors have nothing to disclose.

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