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Photodynamic Therapy with Blended Conducting Polymer/Fullerene Nanoparticle Photosensitizers

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TITLE:
Photodynamic Therapy with Blended Conducting Polymer/Fullerene Nanoparticle Photosensitizers

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SHORT ABSTRACT:
This protocol describes a method for the fabrication of conducting polymer nanoparticles blended with fullerene. These nanoparticles were investigated for their potential use as a next generation photosensitizers for Photodynamic Therapy (PDT).

LONG 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.

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

1.1) 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 minutes. Add 10 ml DMEM media supplemented with 10% FBS to each cell line and centrifuge for 6 min at 106 x g .

1.2) 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 ^{0}C. Label this flask as Passage 0.

1.3) 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.

1.4) Culture the cell lines until passage 11 or 12.

2. Fabrication of nanoparticles

2.1) Preparation of ~10^{-6} M (adjusted) undiluted MEH-PPV stock solution

2.1.1) 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 ^{0}C on a hot plate.

2.1.2) 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.

2.1.3) 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.

2.1.4) 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.

2.1.5) 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.

$$A = \varepsilon \times b \times c \qquad \text{(Eqn 1)}$$

(ε - the molar extinction coefficient, A – absorbance, b – path length, c – concentration of the solution)

$$0.15 = 10^7\ M^{-1}\ cm^{-1} \times 1\ cm \times c \qquad \text{(Eqn 2)}$$

$$c = 1.5 \times 10^{-8}\ M \qquad \text{(Eqn 3)}$$

Use this concentration to find the concentration of the undiluted MEH-PPV stock solution as follows:

$$M_1V_1 = M_2V_2 \qquad \text{(Eqn 4)}$$

$$0.15 \times 10^{-7}\ M \times 3050\ \mu l = M_2 \times 50\ \mu l \qquad \text{(Eqn 5)}$$

$$M_2 = 9.15 \times 10^{-7}\ M \qquad \text{(Eqn 6)}$$

This is the concentration of the 'adjusted undiluted MEH-PPV stock solution'.

2.2) Calculating mass of MEH-PPV in the adjusted undiluted MEH-PPV stock solution

2.2.1) 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 9.15×10^{-4} g.

2.3) Blending PCBM in MEH-PPV

2.3.1) Calculate the mass of Phenyl-C_{61}-butyric acid methyl ester (PCBM) to be added into the stock MEH-PPV solution to make 50wt% PCBM doped MEH-PPV solution, where 50wt% 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/9.15×10^{-4} g of MEH-PPV x 100% = 50wt% PCBM (Eqn 8)
Mass of PCBM = 4.57×10^{-4} g (Eqn 9)

2.3.2) 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 = 2.19×10^{-9} mol/µl (Eqn 11)

2.3.3) Calculate the volume of PCBM solution needed to add to the adjusted undiluted MEH-PPV stock solution to obtain the 50wt% PCBM doped MEH-PPV solution in THF by using the molarity calculated in step 2.3.2

4.57×10^{-4} g of PCBM x 1 mol/910.88 g x 1 µl/2.19×10^{-9} mol = 229 µl (Eqn 12)

2.3.4) Add 229 µl of the PCBM solution into 1 ml of adjusted undiluted MEH-PPV stock solution and mix well.

2.4) Preparation of nanoparticles by reprecipitation method

2.4.1) Transfer 1 ml of the blended MEH-PPV/PCBM solution into the 1 ml syringe with the needle attached to it.

2.4.2) Rapidly inject 1 ml of the blended MEH-PPV/PCBM solution into 4 ml of DI water stirring at 1200 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

3.1) Uptake of nanoparticles in cell lines

3.1.1) 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.

3.1.2) 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.

3.1.3) 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.

3.2) Detection of ROS

3.2.1) 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.

3.2.2) 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.

3.2.3) Warm up the lamp of the solar simulator for 15 minutes. 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.

3.2.4) 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:

$$50\ mW/cm^2 \times 3600\ s/1000 = 180\ J/cm^2$$

3.2.5) 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.

3.2.6) For the positive control incubate the cells with 100 µM hydrogen peroxide (H_2O_2) for 30 min.

3.2.7) 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 ^0C.

3.2.8) 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.

3.3) Apoptosis and necrosis by PI and annexin V FITC

3.3.1) 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.

3.3.2) After 24 hr incubation follow the step 3.2.3.

3.3.3) 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).

3.3.4) Replace the HBSS with DMEM media supplemented with 10% FBS and keep the petri dishes in the incubator for 4 hr.

3.3.5) 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

4.1) Counting cells and culturing in 96-well plates

4.1.1) 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.

4.1.2) 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 x 10^4 cells/ml.

4.1.3) Take 5 96-well plates labeled as 0 hr, 24 hr, 48 hr, 72 hr and 96 hr. Add 50 µl of the 5 x 10^4 cells/ml cell solutions (TE 71 and A549) into the wells as shown in Table 2, thus seeding 2500 cells/well. Do the same procedure for OVCAR3 and MDA-MB-231 cell lines.

4.1.4) 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 x 10^{-4} mg/ml, 2.0 x 10^{-4} mg/ml, and 3.6 x 10^{-4} mg/ml, respectively. Each cell line has triplicates for each concentration of nanoparticles.

4.2) Measuring the cell viability in dark

4.2.1) 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.

4.2.2) Measure the cell viability of the 0 hr plate by recording the absorbance at 570 nm with microplate reader.

4.2.3) 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.

4.2.4) 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.

4.2.5) Measure the cell viability at the particular time points as explained in 4.2.1 and 4.2.2.

4.2.6) Repeat the complete experiment for 3 times (n=3)

5. Measuring cell viability after PDT

5.1) Counting cells and culturing in 96-well plates.

5.1.1) 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.

5.1.2) 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.

5.1.3) After 24 hr add nanoparticles to the plates as explained in 4.1.4.

5.1.4) 24 hr after addition of nanoparticles wash the cells with 1X DPBS and add 50 µl HBSS to each well.

5.1.5) Irradiate the plates with the respective light doses as explained in 3.2.3.

5.1.6) Replace the HBSS with 50 µl DMEM media supplemented with 10% FBS and incubate the plates for the respective time periods after PDT.

5.1.7) After each time period measure the cell viability as explained in 4.2.1 and 4.2.2.

6. Fluorescence microscopy

6.1) Uptake of nanoparticles

6.1.1) 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.

6.1.2) 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.

6.2) Detection of ROS

6.2.1) 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.

6.2.2) 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.

6.3) Apoptosis and necrosis by PI and annexin V FITC

6.3.1) 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.

6.3.2) 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 hours 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 hours 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 hour post-incubation periods. The data are shown in

Figure 3 for the 4 hour 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 LEGENDS:

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).

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.

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).

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.

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).

TABLE LEGENDS:
Table 1: Experimental design for detection of ROS

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

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

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 218W. 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 ^0C (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.

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The authors gratefully acknowledge the National Science Foundation (NSF) for financial support of this work through a CAREER award (CBET-0746210) and through award CBET-1159500. We would like to thank Dr. Turkson (Univ. of Hawaii Cancer Center) and Dr. Altomare (Univ. of Central Florida College of Medicine) for assistance with cell culture.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1 Dolmans, D., Fukumura, D. & Jain, R. K. Photodynamic therapy for cancer. *Nat. Rev. Cancer* **3** (5), 380-387, doi:10.1038/nrc1071 (2003).

2 Dougherty, T. J. *et al.* Photodynamic therapy. *J. Natl. Cancer Inst.* **90** (12), 889-905, doi:10.1093/jnci/90.12.889 (1998).

3 Ferrari, M. Cancer nanotechnology: Opportunities and challenges. *Nat. Rev. Cancer* **5** (3), 161-171, doi:10.1038/nrc1566 (2005).

4 Oleinick, N. L., Morris, R. L. & Belichenko, T. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. *Photochem. Photobiol. Sci.* **1** (1), 1-21, doi:10.1039/b108586g (2002).

5 Ormond, A. & Freeman, H. Dye Sensitizers for Photodynamic Therapy. *Materials* **6** (3), 817-840 doi:10.3390/ma6030817 (2013).

6 Pass, H. I. Photodynamic Therapy in Oncology - Mechanisms and Clinical Use. *J. Natl. Cancer Inst.* **85** (6), 443-456, doi:10.1093/jnci/85.6.443 (1993).

7 Sariciftci, N. S., Smilowitz, L., Heeger, A. J. & Wudl, F. Photoinduced electron transfer from a conducting polymer to buckminsterfullerene. *Science* **258** (5087), 1474-1476, doi:10.1126/science.258.5087.1474 (1992).

8 Sperandio, F. F. *et al.* Photoinduced electron-transfer mechanisms for radical-enhanced photodynamic therapy mediated by water-soluble decacationic C-70 and C84O2 Fullerene Derivatives. *Nanomed-Nanotechnol* **9** (4), 570-579, doi:10.1016/j.nano.2012.09.005 (2013).

9 Fan, J. Q., Fang, G., Zeng, F., Wang, X. D. & Wu, S. Z. Water-Dispersible Fullerene Aggregates as a Targeted Anticancer Prodrug with both Chemo- and Photodynamic Therapeutic Actions. *Small* **9** (4), 613-621, doi:10.1002/smll.201201456 (2013).

10 Grynyuk, I. *et al.* Photoexcited fullerene C-60 disturbs prooxidant-antioxidant balance in leukemic L1210 cells. *Materialwiss. Werkstofftech.* **44** (2-3), 139-143, doi:10.1002/mawe.201300105 (2013).

11 Liu, X. M. *et al.* Separately doped upconversion-C-60 nanoplatform for NIR imaging-guided photodynamic therapy of cancer cells. *Chem. Commun.* **49** (31), 3224-3226, doi:10.1039/c3cc41013g (2013).

12 Trpkovic, A., Todorovic-Markovic, B. & Trajkovic, V. Toxicity of pristine versus functionalized fullerenes: mechanisms of cell damage and the role of oxidative stress. *Arch. Toxicol.* **86** (12), 1809-1827, doi:10.1007/s00204-012-0859-6 (2012).

13 Chen, Z. Y., Ma, L. J., Liu, Y. & Chen, C. Y. Applications of Functionalized Fullerenes in Tumor Theranostics. *Theranostics* **2** (3), 238-250, doi:10.7150/thno.3509 (2012).

14 Park, S. H. *et al.* Bulk heterojunction solar cells with internal quantum efficiency approaching 100%. *Nat Photonics* **3** (5), 297-302, doi:10.1038/nphoton.2009.69 (2009).

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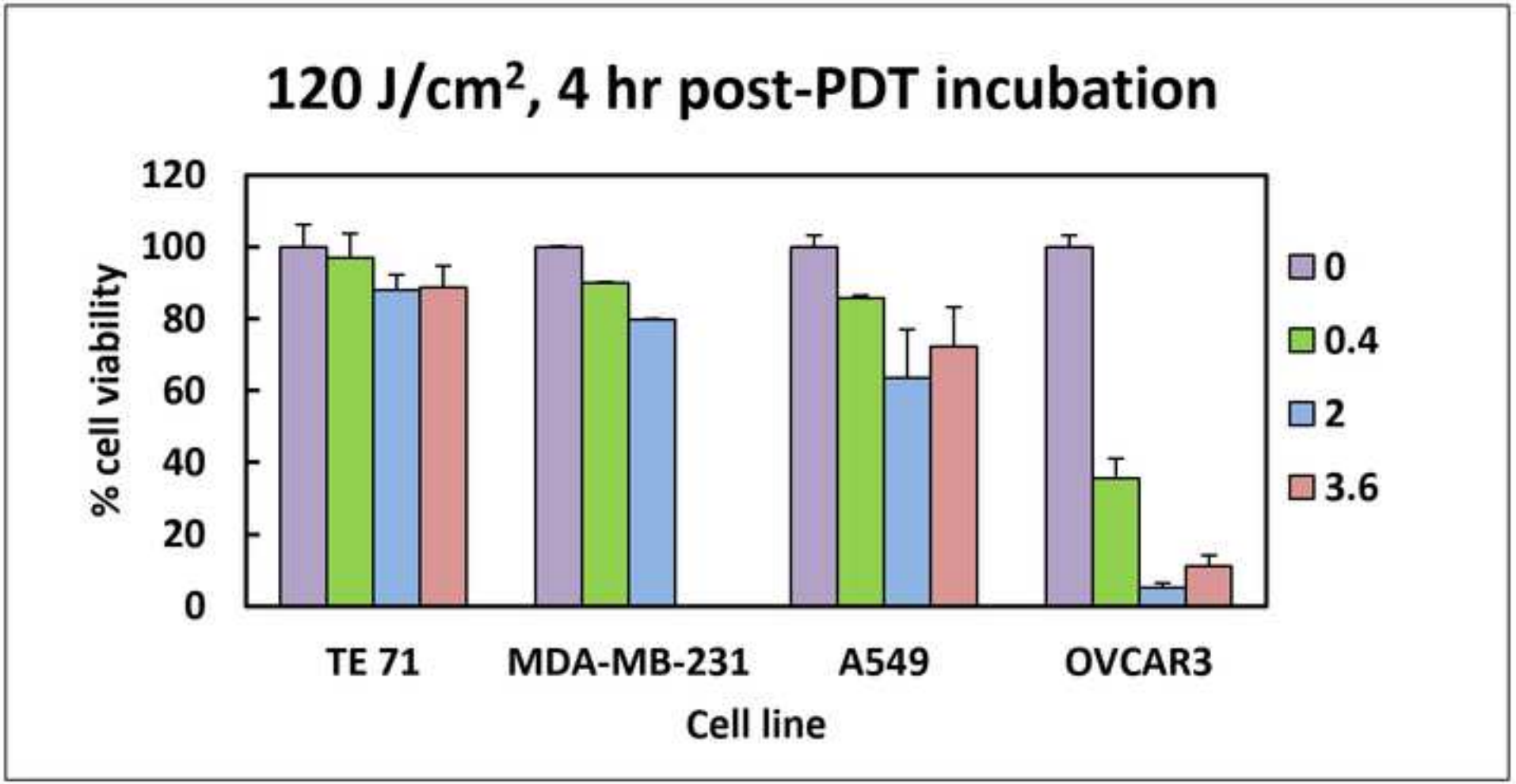

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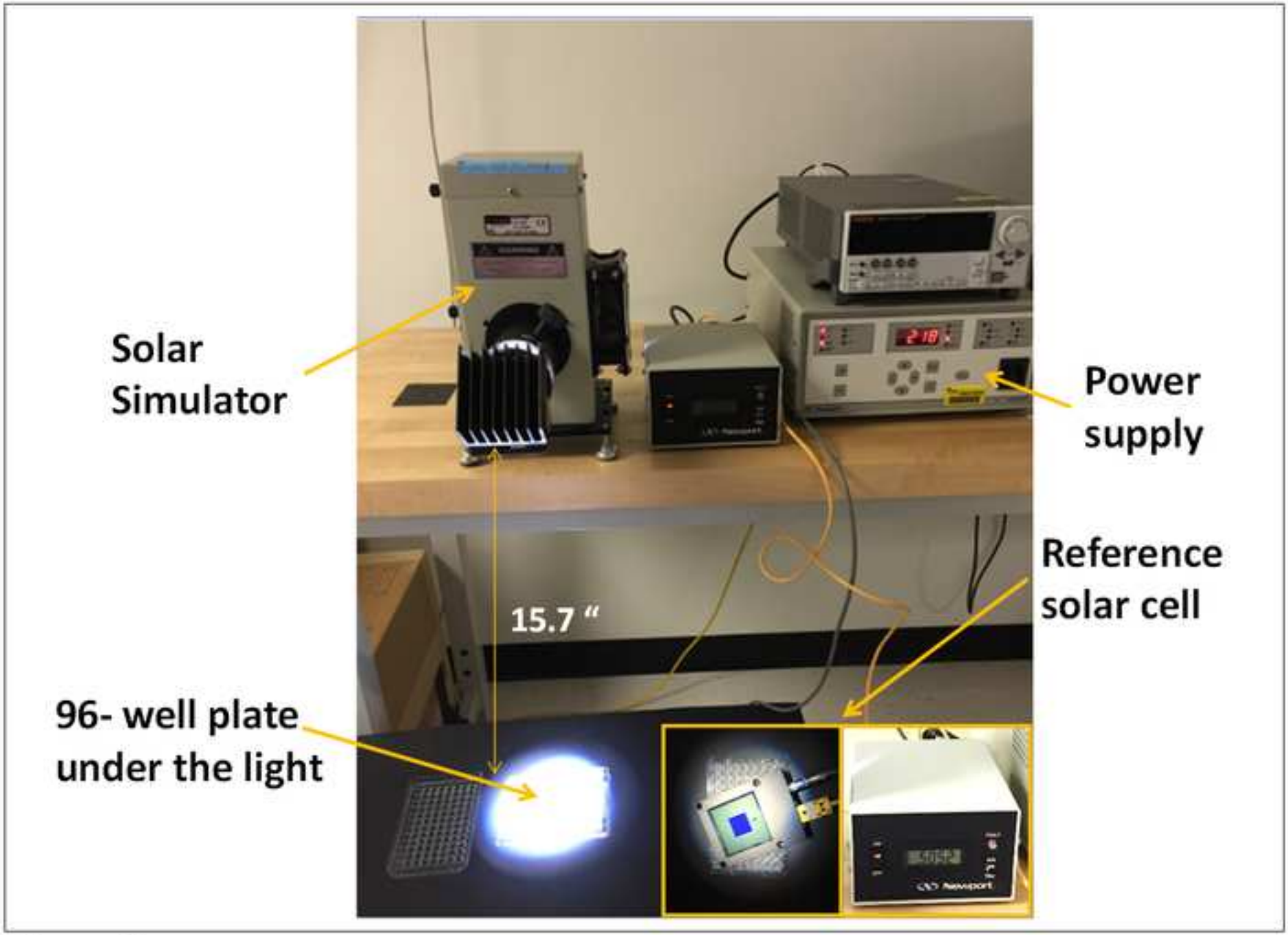

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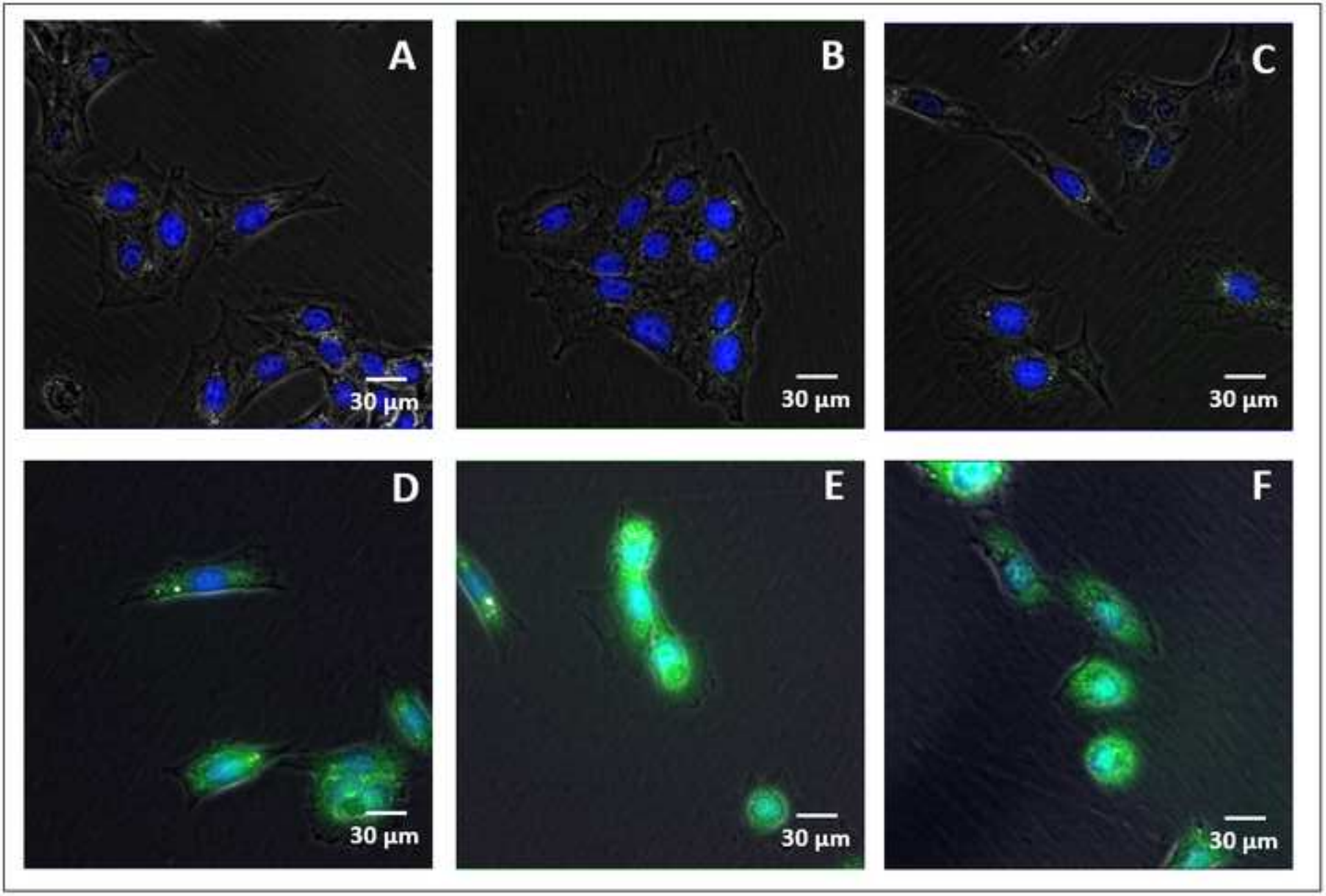

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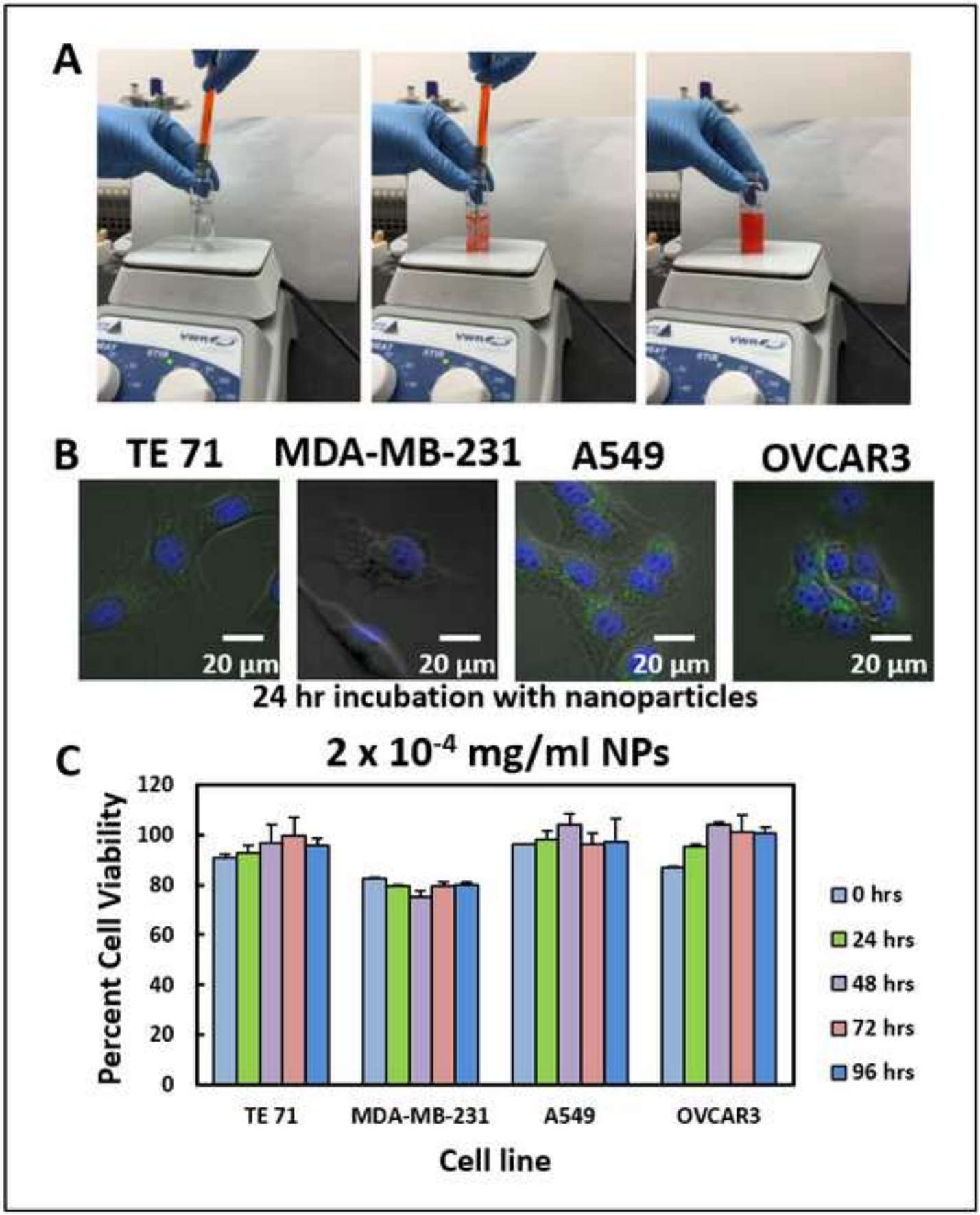

Figure 1

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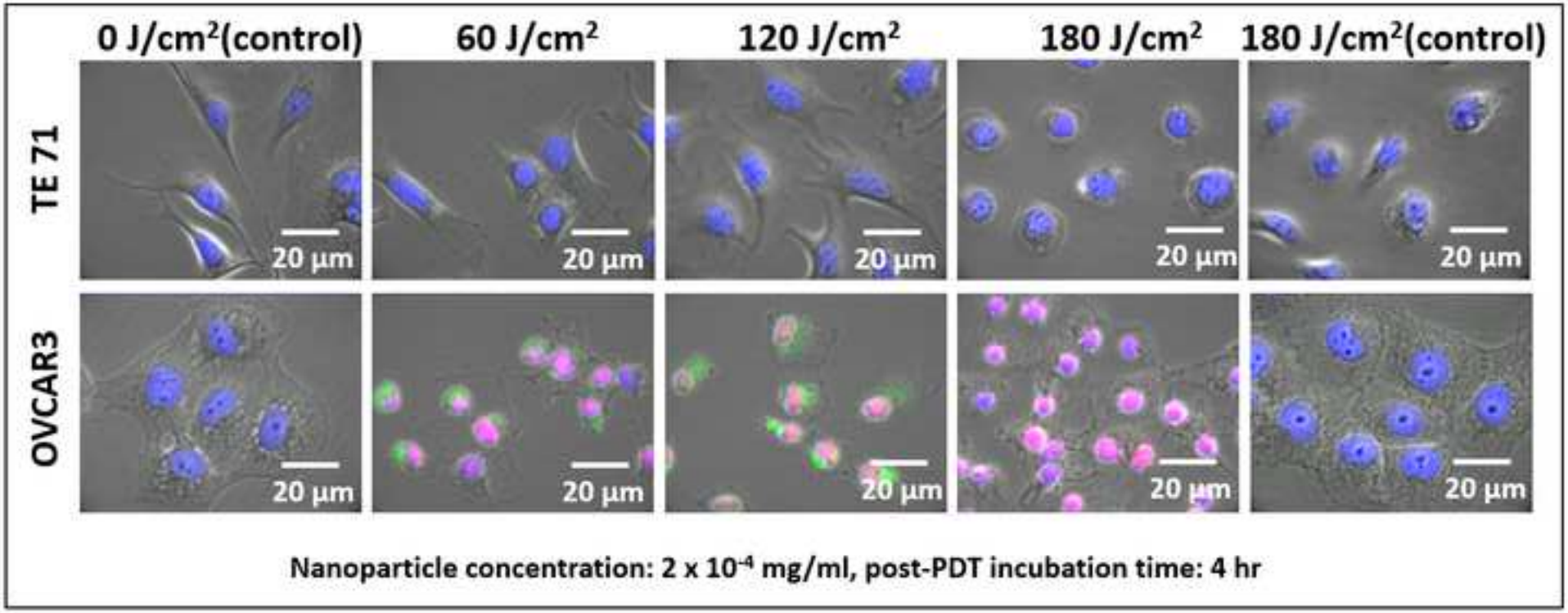

Figure 4

Table 1

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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 2
Click here to download Table: Table 2.xlsx

0 hours plate	Media			TE 71			A549	
No treatment								
0 mg/ml								
0.4×10^{-4} mg/ml								
2.0×10^{-4} mg/ml								
3.6×10^{-4} mg/ml								

	Media		

Table 3

Click here to download Table: Table 3.xlsx

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 4
Click here to download Table: Table 4.xlsx

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

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: gesquiere JoVE_Materials2.xls

Name of Material/ Equipment	Company	Catalog Number
Poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV)	Sigma Aidrich	536512-1G
[6,6]-Phenyl C61 butyric acid methyl ester (PCBM)	Sigma Aidrich	684449-500MG
Tetrahydrofuran (THF)	EMD	TX0284-6
1 ml syringe	National Scientific Company	37510-1
Syringe filter	VWR	28145-495
1 ml syringe	Hamilton Company	81320
Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 Mix (DMEM)	Corning (VWR)	45000-350
Hank's Balanced Salt Solution without phenol red (HBSS)	Quality Biological (VWR)	10128-740
Dulbecco's Phosphate-Buffered Saline, 1X without calcium and magnesium (DPBS)	Corning (VWR)	45000-436
Fetal Bovine Serum, Regular (Heat Inactivated) (FBS)	Corning (VWR)	45000-736
Trypsin EDTA 1X 0.25%	Corning (VWR)	45000-664
16% Paraformaldehyde	Electron Microscopy Sciences	15710
DAPI	Biotium VWR	89139-054
5 ml pipettes	VWR	82050-478
75 cm^2 culture flask	VWR	82050-856
96-well plates	VWR	82050-771
Tissue Culture Dishes with Vents	Greiner Bio-One (VWR)	82050-538
Propidium iodide	Molecular probes	P3566

Annexin V FITC	Invitrogen	A13199
Celltiter 96 non-R 1000 assays	Promega (VWR)	PAG4000
CellROX Green Reagent, for oxidative stress detection	Invitrogen	C10444
UV-vis spectrometer	Agilent 8453	
Fluorescence spectrometer	NanoLog HoribaJobin Yvon	
Dynamic light scattering	PD2000DLS, Precision detector	
Incubator	NuAir DH Autoflow	
Confocal microscope	Zeiss Axioskop2	
Epiluminescence microscope	Olympus IX71	
Solar Simulator	Newport 67005 Oriel Instruments	
Reference solar cell	Oriel	
Microplate reader	BioTek Ex808	
Hemocytometer	Hausser Scientific Partnership	3200

Comments/Description

average M_n 150,000-250,000

> 99.5%

Drisolv

For filtration of MEH-PPV solution

25 mm, 0.2 µm, PTFE
For injection of MEH-PPV solution into water to make nanoparticles

Trypsin/2.21 mM EDTA in HBSS without sodium bicarbonate, calcium and magnesium Porcine Parvovirus Tested

16% paraformaldehyde is diluted to 4% by adding PBS

Nuclear stain

for culturing cells
for MTT assays

dye for apoptosis
MTT

For ROS detection

63X oil immersion objective lens
60X water immersion objective lens, Andor Zyla sCMOS camera

VLSI Standards Incorporated

For counting cells

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Photodynamic Therapy with Blended Conducting Polymer/Fullerene Nanoparticle Photosensitizers

The authors thank the referees for their thorough review, which has helped to significantly improve the quality of our manuscript.

Editorial comments:

A number of grammar/typo issues need to be addressed:

Please fix the grammar and typographical errors in both abstracts.

Please copyedit the manuscript to remove frequent errors grammar and in spacing. These errors typically occur after a unit of time.

Line 327: Preferential what of nanoparticles?

Answer: Preferential uptake of nanoparticles

2. There are some formatting issues to be corrected:

PDT should be defined at first mention (Line 55), not second (Line 65).

Answer: 'Photodynamic Therapy' added on line 55 and deleted from line 65

Are there better descriptors for the solvents to replace "good" and "bad?"

Answer: The good and bad words are replaced by compatible and non-compatible respectively.

Please define HBSS, PI

Answer: HBSS: Hank's Balanced Salt Solution, and PI: Propidium iodide

5.1.7: Please cite steps by step name (eg. 4.2.1)

Answer: Steps are now cited by name

References: Please make sure all journal titles are abbreviated.

Answer: All journal titles are now abbreviated

3. Addtitional detail is required:

1.1: Please provide a reference on how these cells are cultured, or else provide more stepwise detail.

Answer: More stepwise detail has been provided in the paper, marked here as yellow highlight

1.1) 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 minutes. Add 10 ml DMEM media supplemented with 10% FBS to each cell line and centrifuge for 6 min at 1000 rpm.

1.2) Remove 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 ^{0}C. Label this flask as Passage 0.

1.3) 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 1000 rpm. 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.

1.4) Culture the cell lines until passage 11 or 12.

2.1.3: What wavelength is used?

Answer: The wavelength used is 495 nm, added in the paper.

2.4.2: Are the nanoparticles processed after injection?

Answer: The nanoparticles are not processed after injection. Statement added to paper

3.3.5: How much annexin V FITC and PI?

Answer: 20 µl annexin V FITC was used from the purchased stock solution. 300 nM PI was used. Details added to paper.

Figure legends: Please provide titles for Fig. 1 & 3. Also, please define the error in these figures.

Answer: The titles for Figure 1 and 3 are: Figure 1A- Fabrication of nanoparticles, Figure 1B: Uptake of nanoparticles by different cell lines, Figure 1C: Intrinsic cytotoxicity of nanoparticles, Figure 3: Cell viability after PDT.

The error in Figure 1C and in Figure 3 are the standard deviations for results from 3 separate experiments (n=3). Statement added to respective figure captions.

4. There is unnecessary branding in step 3.2.3: FSQ-GG400.

Answer: FSQ-GG400 is deleted.

5. Discussion: What are the limitations of this protocol?

Answer: Limitations of this protocol:

1) The nanoparticle size is hard control due to the preparation method used. As the injection of solution to make nanoparticles is performed manually with hands, the injection rate is hard to control from person to person. This can pose as a limitation to this protocol.
2) During PDT the cells have to be kept outside the incubator for several hours which imposes some stress on the cells.

Statements were added to the discussion section of the paper.

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Answer: DOI added to all the references.

Reviewer 1:

No manuscript summary

No major comments

Minor comments:

1. **Page 1, lines 36-37: "... the fabrication of blended conducting polymer nanoparticles blended with fullerene as the next generation photosensitizers ..."**
   Could the first "blended" be deleted?
   Answer: first 'blended' is deleted
2. **Page 1, line 48: " ... and efficient and charge and energy transfer to the fullerene PCBM ..."**
   Delete the second "and".
   Answer: The second 'and' is deleted
3. **Page 2, lines 59-61: "... however, 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 remain..."**
   This sentence seems problematic in term of grammar.
   Answer: sentence was split and further edited.
4. **Page 2, lines 67-68: "... when these materials dissolved in a good solvent are rapidly injected into a bad solvent ..."**
   Please reword this sentence.
   Answer: The words compatible-non-compatible solvents are used instead of good and bad solvents.
5. **Page 4, line 183, Section 3.2.3**
   Is a power meter used for measuring the light irradiance here?
   It would better to measure the irradiance at the surface of the cell culture instead of the surface of the pertri dish.
   Answer: A calibrated reference photosensor was used to measure the light irradiance.
   The distance between surface of cell culture and surface of petri dish is negligible and the irradiance was not observed to change at these small distances.
6. **Page 5, lines 185-186: "... the surface of the petri dish/96-well plate..."**
   The authors may want to delete "/96-well plate", because in this section only petri dishes are used.
   Answer: '/96 well plate' is deleted

Reviewer 2:

No comments

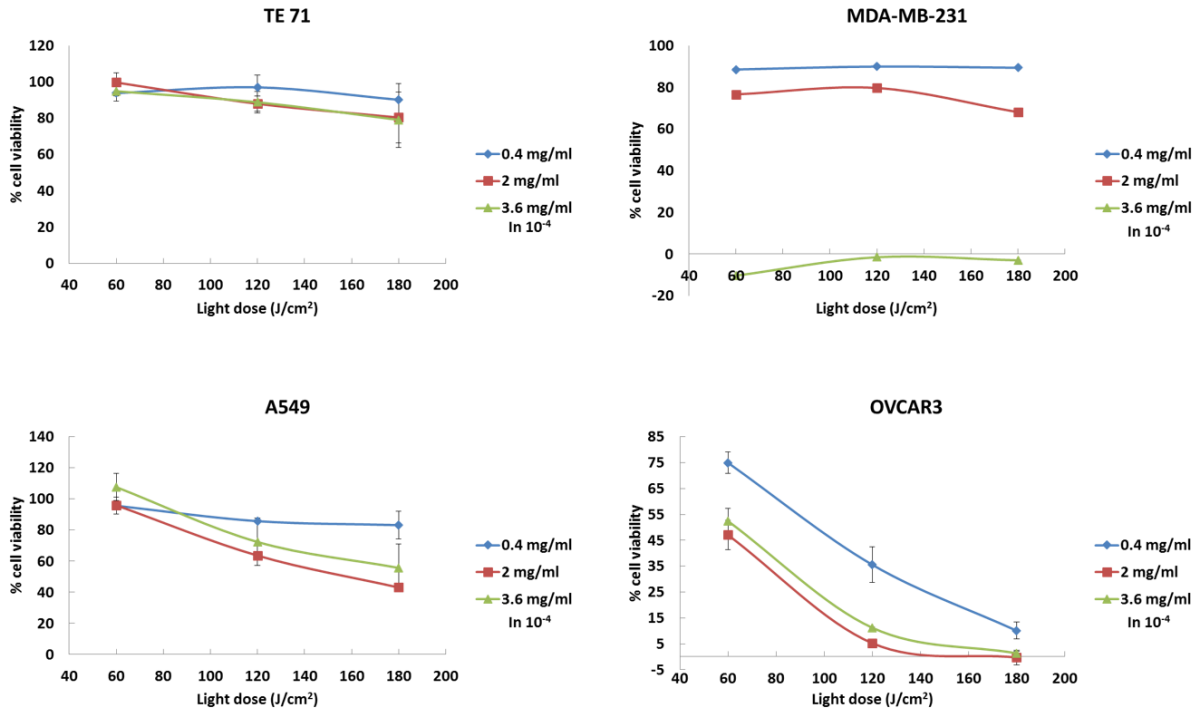
Reviewer 3:

Manuscript summary:

This paper describes the use of a semi-conducting polymer (MEH-PPV) to solubilize a C60 fullerene derivative (phenyl c61 butyric acid methyl ester) to act as a photosensitizer to mediate PDT killing of 3 cancer and 1 normal cell line.

Major comments:

1. **They claim that the polymer acts as "high extinction coefficient that leads to high rates of triplet formation, and both efficient and ultrafast charge and energy transfer to the fullerene" but no evidence is presented to back up this claim, only a citation to a paper.**
   The study of charge and energy transfer from MEH-PPV to PCBM was not performed in our lab but has been extensively reported in literature since the early nineties. Further study would add no new information to the existing body of work.
2. **It is very premature to claim that the complex "shows unexpected specificity to cancer cell lines. " They basically have 1 "normal cell" and 3 cancer cells. Only the OVCAR3 cell is highly susceptible to PDT (see figure 3). This might be explained by the fact that OVCAR3 is a non-adherent cell line while the other cells are adherent. In order to substantiate the claim for specificity for cancer cells they would need at least 3 of each with comparable levels of adherence**.
   Cell adherence was verified by phase imaging and was found to be excellent and consistent across the cell lines studied here. The difference in PDT efficiency is due to differences in extent of nanoparticle uptake. The ROS formation is directly proportional to amount of nanoparticles uptaken. The uptake of nanoparticles depends on the metabolic rate of the cell lines and it is well established that OVCAR3 has a high metabolic rate compared A549 or MDA-MB-231. Figure 1 supports that OVCAR3 has taken more nanoparticles.
3. **In order to demonstrate in vitro PDT they need to carry out a light-dose response experiment as well as the PS dose response experiment shown in Figure 3.**
   We have carried out the light-dose response experiments as the referee suggests. We have used 60, 120 and 180 J/cm^2 light doses and observed that the PDT effect increases with increasing light dose. We opt to not include these data in order to keep the paper sufficiently brief. Furthermore, the methods described herein will not be further developed by including this information. The Figure below shows cell viability as a function of light dose for each cell line, plotted for each nanoparticle dose.



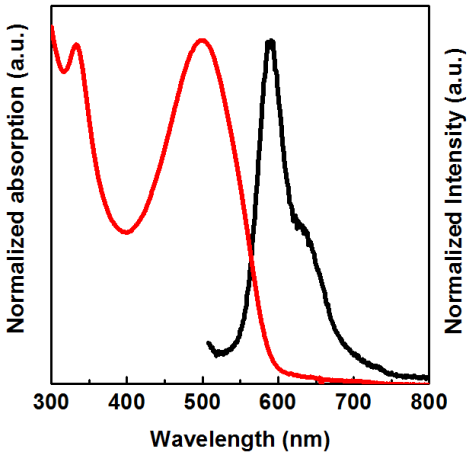
4. **Apparently they used the CellRox kit to determine ROS produced inside cells. The Methods section does not give details of how this kit was used. However I believe what is happening is that the kit is detecting ROS produced from mitochondria in the PDT damaged cells NOT ROS produced from the PDT. This can be seen from the fact that there is higher fluorescence at 2 hours after light delivery than there is immediately (see Figure 2).**

   CellRox is received as a concentrated solution. The methods section describes the appropriate dilution into culture media, the incubation time and incubation temperature. No further steps are necessary for this assay.

   The data in Figure 2 show that the ROS are formed from the nanoparticles. Panel B shows the control experiment where the samples went to the full PDT treatment, but in the absence of nanoaparticles. In that case no ROS formation is detected, which eliminates the hypothesis of detecting ROS produced directly from mitochondria. Although we cannot fully eliminate that mitochondria in dying /dead cells have released ROS, CellRox emission is detected throughout the whole cell body. Based on these two observations it is reasonable to speculate that most of the observed ROS formation is due to the nanoparticles, and that the difference between panels D and E is more likely due to a heterogeneous distribution of nanoparticles across different cells. In addition, lipid peroxidation reaction could continue during the 2 hour period since this is basically a continuously propagating radical chemistry, which can further explain the observations in panel E.

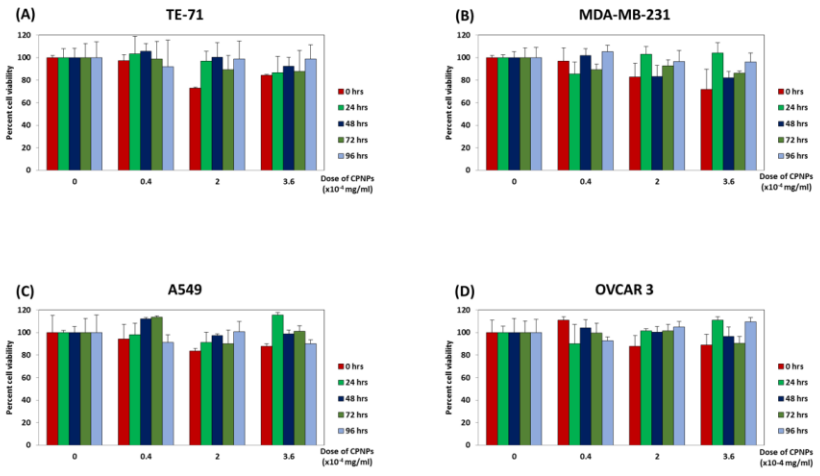
5. **It is not clear what the fluorescence of the nanoparticles in the cells in Figure 1 is actually due to. There is no excitation and emission details given and no fluorescence of the prepared nanoparticles is given. Presumably the MEH-PPV as the fullerene has no fluorescence?**

The fluorescence of nanoparticles in the cells in Figure 1 is due to MEH-PPV emission at 589 nm. The excitation and emission details are given by the graph shown below:



Minor comments:

1. **It is not correct to state regarding fullerenes "However, severe cytotoxicity has hampered further development". By and large fullerenes are considered only of low toxicity compared to other classes of nanomaterials. There can be some toxicity depending on the functionalization and delivery vehicle, but it is not clear that the MEH-PPV has any effect on the dark cytotoxicity.**
   Per the referees suggestion we have completed dark cytotoxicity studies on undoped MEH-PPV nanoparticles, which show that MEH-PPV has no statistically significant effect on dark toxicity. Please see the Figure below. Fullerene cytotoxicity has been reported in literature, although there are broad discrepancies in the reported dose dependencies. A couple of representative references are include below.



(1) Sayes, C. M.; Gobin, A. M.; Ausman, K. D.; Mendez, J.; West, J. L.; Colvin, V. L. Nano-C-60 cytotoxicity is due to lipid peroxidation. *Biomaterials* **2005**, *26*, 7587-7595.

(2) Zhang, B.; Bian, W. L.; Pal, A.; He, Y. L. Macrophage apoptosis induced by aqueous C-60 aggregates changing the mitochondrial membrane potential. *Environ. Toxicol. Pharmacol.* **2015**, *39*, 237-246.

2. **The statement "AFM was not used for size determination as during the sample preparation the nanoparticles become flat due to vacuum drying." is somewhat strange. What is going on here? Moreover TEM is universally used for characterizing nanoparticles.**
   The nanoparticles are soft and spherical in suspension. When vacuum is applied to dry the sample for AFM imaging, the nanoparticles collapse and become disc like. The AFM tip was also found to push down on the nanoparticles. Hence AFM was not used in this study. This was mentioned as an additional guide to readers, but as the sentence appears confusing we have deleted it from the manuscript.

Reviewer 4:

No manuscript summary

Major comments:

The authors explained the differences in PDT results between the cancer cell lines by the difference in aggressiveness (metabolism and rate of endocytosis) between these cell lines. But how to explain that both mouse thymic epithelial (not cancer and not aggressive) cells and human breast cancer cells (cancer and aggressive) are not sensitive to PDT?
The aggressiveness (metabolism and rate of endocytosis) of the cell lines directly determines the extent of uptake of nanoparticles by each cell lines. The aggressive cell lines (OVCAR3 followed by A549) take up high amounts of nanoparticles. ROS formation can be assumed to be relatively proportional to the amount of nanoparticles available. Thus, higher levels of ROS are generated for OVCAR3 and A549, resulting in better PDT efficacy. On the other hand MDA-MB-231 is not as aggressive and takes up less nanoparticles. TE71 is a normal control and should not take nanoparticles, and should not be showing PDT effects.

Minor comments:
In legend to Fig. 1B the time of cells incubation with nanoparticles and in legend to Fig. 4 the nanoparticles concentration and the time after photoexcitation should be indicated.
Legends to figure 1B and figure 4 added.