Video Article

Electroactive Polymer Nanoparticles Exhibiting Photothermal Properties

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Abstract

A method for the synthesis of electroactive polymers is demonstrated, starting with the synthesis of extended conjugation monomers using a three-step process that finishes with Negishi coupling. Negishi coupling is a cross-coupling process in which a chemical precursor is first lithiated, followed by transmetallation with $ZnCl_2$. The resultant organozinc compound can be coupled to a dibrominated aromatic precursor to give the conjugated monomer. Polymer films can be prepared via electropolymerization of the monomer and characterized using cyclic voltammetry and ultraviolet-visible-near infrared (UV-Vis-NIR) spectroscopy. Nanoparticles (NPs) are prepared via emulsion polymerization of the monomer using a two-surfactant system to yield an aqueous dispersion of the polymer NPs. The NPs are characterized using dynamic light scattering, electron microscopy, and UV-Vis-NIR-spectroscopy. Cytocompatibility of NPs is investigated using the cell viability assay. Finally, the NP suspensions are irradiated with a NIR laser to determine their effectiveness as potential materials for photothermal therapy (PTT).

Video Link

The video component of this article can be found at http://www.jove.com/video/53631/

Introduction

Electroactive polymers change their properties (color, conductivity, reactivity, volume, *etc.*) in the presence of an electric field. The rapid switching times, tunability, durability, and lightweight characteristics of electroactive polymers have led to many proposed applications, including alternative energy, sensors, electrochromics, and biomedical devices. Electroactive polymers are potentially useful as flexible, light-weight battery and capacitor electrodes.[1] Applications of electroactive polymers in electrochromic devices include glare-reduction systems for buildings and automobiles, sunglasses, protective eyewear, optical storage devices, and smart textiles.[2-5] Smart windows can reduce energy requirements by blocking specific wavelengths of light on-demand and protecting interiors of homes and automobiles. Smart textiles can be used in clothing to help protect against UV radiation.[6] Electroactive polymers have also begun to be used in medical devices. Among electroactive polymers used in biomedical devices, polypyrrole (PPy), polyaniline (PANI), and poly(3,4-ethylenedioxythiophene) (PEDOT) are among the most common. For example, these types of polymers are commonly used as transducers in biosensor devices.[7] Applications in therapeutic delivery have also shown promise; studies have demonstrated the release of drugs and therapeutic proteins from devices prepared from electroactive polymers.[8-12] More recently, electroactive polymers have been used as therapeutic agents in photothermal therapy.[13-15] In photothermal therapy, photothermal agents must absorb light in the near-infrared (NIR) region (~ 700−900 nm), also known as the therapeutic window, where light has the maximum depth of penetration in tissue, typically up to 1 cm.[16,17] In this range, biological chromophores such as hemoglobin, oxygenated hemoglobin, lipids, and water have little-to-no absorbance, which enables light to penetrate easily. When photothermal agents absorb light in this therapeutic window, the photoenergy is converted to photothermal energy.

Irvin and co-workers have previously reported alkoxy-substituted bis-EDOT benzene monomers that were synthesized using Negishi coupling.[18] Negishi coupling is a preferred method for carbon-carbon bond formation. This process has many advantages, including the use of organozinc intermediates, which are less toxic and tend to have higher reactivity than other organometallics used.[19,20] Organozinc compounds are also compatible with a wide range of functional groups on the organohalides.[20] In the Negishi coupling reaction, an organohalide and organometal are coupled through the use of a palladium(0) catalyst.[20] In the work presented herein, this cross coupling method is utilized in the synthesis of 1,4-dialkoxy-2,5-bis(3,4-ethylenedioxythienyl) benzene (BEDOT-B$(OR)_2$) monomers. These monomers can then be easily polymerized electrochemically or chemically to yield polymers that are promising candidates for use in biomedical applications.

Conventional methods for preparation of colloidal polymeric suspensions in aqueous solutions for biomedical applications typically involve the dissolution of bulk polymers followed by nanoprecipitation or emulsion-solvent evaporation techniques.[21,22] In order to produce NPs of poly(BEDOT-B$(OR)_2$), a bottom-up approach is demonstrated here where the NPs are synthesized via *in situ* emulsion polymerization. Emulsion polymerization is a process that is easily scalable and is a relatively fast method for NP preparation.[22] Studies using emulsion polymerization

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to produce NPs of other electroactive polymers have been reported for PPy and PEDOT.[15,23,24] PEDOT NPs, for example, have been prepared using spray emulsion polymerization.[24] This method is difficult to reproduce, and typically yields larger, micron-sized particles. The protocol described in this article explores the use of a drop-sonication method to reproducibly prepare 100-nm polymer NPs.

In this protocol, electroactive polymers tailored to absorb light in the NIR region similar to previously reported poly(BEDOT-B$(OR)_2$) are synthesized and characterized to demonstrate their potential in electrochromic devices and as PTT agents. First, the protocol for the synthesis of the monomers via Negishi coupling is described. The monomers are characterized using NMR and UV-Vis-NIR spectroscopy. The preparation of NP colloid suspensions via oxidative emulsion polymerization in aqueous media is also described. The procedure is based on a two-step emulsion polymerization process previously described by Han *et al.* that is applied to the different monomers. A two-surfactant system is used to control the NP monodispersity. A cell viability assay is used to evaluate cytocompatibility of the NPs. Lastly, the potential of these NPs to act as PTT transducers is demonstrated by irradiation with a NIR laser.

Protocol

Caution: Please consult all relevant Safety Data Sheets (SDS) before use. Several of the reagents used in these syntheses are potentially hazardous. Please use all appropriate safety practices including personal protective equipment (safety glasses, gloves, lab coat, long pants, and closed-toe shoes), and perform syntheses in fume hoods. Lithiation is particularly hazardous and should only be performed by properly trained individuals with supervision.

1. Monomer Synthesis

Note: **Figure 1** shows the chemical route for the preparation of precursors and monomers whose synthesis is described in Sections 1.2 - 1.5.

1. Materials
    1. Purify EDOT as described previously.[25]
    2. Recrystallize tetrabutyl ammonium perchlorate (TBAP) from ethyl acetate and dry under vacuum for 24 hr. Titrate n-butyllithium (nBuLi, 2.5 M in hexanes) as described by Hoye *et al.*[26] within 48 hr prior to use to determine the actual concentration.
    3. Dry magnesium sulfate and potassium carbonate at 100 °C for 24 hr prior to use. Use all other chemicals used in this protocol as received.

2. Synthesis of 1,4-Dialkoxybenzenes
Note: **Figure 1A** shows the preparation of 1,4-dihexyloxybenzene using 1-bromohexane.
    1. Equip an oven-dried three-neck round bottom flask with a septum, an argon inlet adapter, and a condenser fitted with a gas outlet adapter connected to a bubbler. Add a stir bar to the flask prior to sealing.
    2. Connect the inlet adapter to a Schlenk line using poly(vinyl chloride) (PVC) tubing and purge the round bottom flask with argon.
    3. Add 12.5 g (113.5 mmol) of hydroquinone to the round bottom flask and dissolve it in 20 ml of anhydrous tetrahydrofuran (THF) with stirring.
    4. Separately, dissolve 14 g (250 mmol) of KOH in 30 ml of ethanol in a single-neck round bottom flask and stir until dissolved.
    5. Once dissolved, slowly add the KOH solution to the three-neck round bottom flask using a syringe. Allow the mixture to stir for 1 hr.
    6. After 1 hr, add 250 mmol of 1-bromoalkane to the reaction mixture.
    7. Heat the reaction mixture at reflux for 24 hr with stirring under argon.
    8. After 24 hr, allow the reaction mixture to cool to RT and add 15 ml DI water and 10 ml of dichloromethane.
    9. Transfer the mixture to a separatory funnel. Isolate the organic layer and wash it three times with 10 ml of DI water.
    10. Dry the organic layer over 15 g of $MgSO_4$ for 15 min.
    11. Remove the $MgSO_4$ via vacuum filtration through filter paper.
    12. Remove the solvent from the filtered solution using a rotary evaporator at 50 °C and 21 kPa to yield 1,4-dialkoxybenzene as a crude white solid.
    13. Recrystallize the crude product by adding just enough hot ethanol to dissolve the product. Once dissolved, place in an ice bath to induce crystallization.
    14. Collect crystals via vacuum filtration through filter paper and wash with cold ethanol.
    15. Dry the crystals under vacuum for 24 hr at RT and store them under argon until further use. This procedure produces 1,4-dihexyloxybenzene.
    16. Characterize the product using melting point and ^{1}H and ^{13}C NMR spectroscopy.[27]

3. Synthesis of 1,4-Dialkoxybenzenes containing ester moieties
Note: **Figure 1B** shows the chemical route for the preparation of a 1,4-dialkoxybenzene using ethyl-4-bromobutanoate.
    1. Equip an oven-dried three-neck round bottom flask with a septum, an argon inlet adapter, and a condenser fitted with a glass outlet adapter connected to a bubbler. Add a stir bar to the flask prior to sealing.
    2. Connect the inlet adapter to the Schlenk line using PVC tubing and purge with argon.
    3. Weigh 1.88 g (93.5 mmol) of KI and 15.69 g (93.3 mmol) of K_2CO_3 and add to the round bottom flask.
    4. Add 25 ml of anhydrous *N,N*-dimethylformamide (DMF) and stir until the salts dissolve.
    5. Once dissolved, add 2.5 g (18.7 mmol) of hydroquinone to the reaction mixture and allow the reaction to stir until dissolved.
    6. When all solids are dissolved, add 46.8 mmol of alkyl bromoalkanoate; heat the reaction mixture at reflux for 24 hr under argon with continuous stirring.
    7. Remove the reaction mixture from heat and allow it to cool to RT.
    8. Transfer the reaction mixture to a separatory funnel and add water (20 ml) and ethyl acetate (20 ml) to extract the organic layer. Isolate the organic layer and wash it three times with water (20 ml portions).
    9. Dry the organic layer over 15 g of $MgSO_4$ for 15 min. Once dried, remove $MgSO_4$ from the mixture via vacuum filtration through filter paper.

10. Remove the solvent using a rotary evaporator at 100 °C and 21 kPa. Dry the crude product under vacuum at RT O/N.
11. Recrystallize the product by adding just enough hot ethanol to dissolve all the solid. Once dissolved, cool the flask in ice and allow crystals to form. Collect the product via vacuum filtration and wash with cold ethanol.
12. Dry the crystals under vacuum at RT for 24 hr and store under argon until further use. This procedure produces 1,4-bis(ethyl butanoyloxy)benzene.
13. Characterize the product using melting point and ^{1}H and ^{13}C NMR spectroscopy.[28]

4. Synthesis of 1,4-Dialkoxy-2,5-dibromobenzenes
Note: The chemical route for the preparation of 1,4-dialkoxy-2,5-dibromobenzenes is shown in **Figure 1A** and **1B**.
1. Fit a dry, three-neck round bottom flask with an argon inlet, a constant pressure addition funnel capped with a glass stopper or septum, and an outlet connected to plastic tubing fitted with an inverted glass funnel suspended over a 1 M NaOH solution.
2. In this round bottom flask, dissolve 218 mmol of 1,4-dialkoxybenzene in dichloromethane (15 ml).
3. Separately, add 12 ml (598 mmol) of Br_2 to a 250 ml flask and dilute with dichloromethane (12 ml).
4. Transfer the Br_2/dichloromethane solution to the constant pressure addition funnel. Add the Br_2 solution dropwise into the three-neck round bottom flask with stirring under argon over a span of 2 hr.
5. After complete addition, allow the reaction to stir O/N under continuous argon flow.
6. Quench the reaction by adding DI water (20 ml), and pour the mixture into a separatory funnel.
7. Isolate the organic layer and wash three times with DI water (20 ml portions). Dry the organic layer over 15 g of $MgSO_4$ for 15 min.
8. Remove the $MgSO_4$ by vacuum filtration through filter paper, and remove the solvent using a rotary evaporator at 75 °C and 21 kPa.
9. Purify crude 1,4-dialkoxy-2,5-dibromobenzene by adding just enough hot ethanol to dissolve all the solid. Once dissolved, cool the flask in ice and allow crystals to form. Collect the product via vacuum filtration and wash with cold ethanol.
10. Dry the purified product under vacuum at RT O/N; store under argon.
11. Characterize the product using melting point and ^{1}H and ^{13}C NMR spectroscopy.[27,28]

5. Negishi Coupling of 1,4-Dialkoxy-2,5-dibromobenzenes with 3,4-Ethylenedioxythiophene (EDOT)
Note: **Figure 1C** shows the Negishi coupling of 1,4-dialkoxy-2,5-dibromobenzenes with EDOT to form monomers M1 and M2.
1. Fit a clean three-neck round bottom flask with a septum, a condenser fitted with an inlet flow control adapter connected to argon, and a gas outlet flow control adapter connected to a bubbler.
2. Connect the inlet adapter to the Schlenk line using thick-walled PVC tubing. Begin flowing argon into the reaction flask for several minutes.
3. Using a Bunsen burner, flame-dry the apparatus under vacuum and purge with argon three times in order to ensure an airless environment.
4. Weigh 1.07 g (10 mmol) of purified EDOT and add to the reaction flask using a syringe inserted through the septum. Dilute the EDOT with anhydrous THF (20 ml) and stir under argon.
5. Chill the flask containing the EDOT solution using a dry ice/acetone bath for 15 min at −78 °C.
6. After 15 min, slowly add 11 mmol nBuLi in hexanes solution dropwise while maintaining the temperature at -78 °C. Stir the reaction at -78 °C for 1 hr.
Note: The exact concentration of the nBuLi should be determined by titration prior to use as per Section 1.1.
7. After 1 hr of stirring, remove the dry ice/acetone bath.
8. Immediately after removal of the bath, add 14.13 ml of 1.0 M $ZnCl_2$ solution dropwise. Allow the reaction to proceed for 1 hr while stirring at RT.
9. After 1 hr of stirring, add 4 mmol of 1,4-dialkoxy-2,5-dibromobenzene and 0.08 mmol of tetrakis(triphenylphosphine)palladium(0) to the reaction mixture.
10. Heat the reaction mixture at reflux (70 °C) in an oil bath.
11. Track reaction progress using thin layer chromatography (TLC): Take small (0.2 ml) aliquots of the reaction mixture daily using a syringe and precipitate into 2 ml 1 M HCl. Extract with 2 ml $CHCl_3$ and spot the extract on a silica TLC plate alongside spots of solutions of EDOT and the appropriate1,4-dialkoxy-2,5-dibromobenzene. Elute with 60:40 ethyl acetate:hexane.
12. When the reaction is complete, allow the reaction mixture to cool to RT. Quench the reaction by adding 10 ml of 1 M HCl followed by the addition of dichloromethane (20 ml).
13. Transfer to a separatory funnel and isolate the organic layer.
14. Wash the organic layer with DI water until the wash water is no longer acidic. Test the acidity of the wash water with pH paper.
15. Dry the organic layer over 15 g of $MgSO_4$, filter, and remove solvent using a rotary evaporator at 50 °C and 21 kPa to yield the crude extended conjugation monomer (M1 or M2) as a yellow-orange solid.
16. Recrystallize the crude product using a hot solution of 3:1 ethanol:benzene solution for M1 or 7:2 hexane:benzene for M2. Add just enough hot solvent mixture to dissolve the solid. Once dissolved, cool the flask in ice and allow crystals to form. Collect the product via vacuum filtration and wash with cold ethanol.
17. Dry the product under vacuum for 24 hr at RT. Store in the dark under argon.
18. Characterize the product using melting point and ^{1}H and ^{13}C NMR spectroscopy.[18]

2. Electrochemistry

1. Electropolymerization
1. In a 50 ml volumetric flask prepare a 100 mM tetrabutylammonium perchlorate (TBAP) electrolyte solution in anhydrous acetonitrile (CH_3CN).
2. In a 10 ml volumetric flask prepare a 10 mM monomer (M1 or M2) solution using the 100 mM TBAP/CH_3CN solution as diluent.
3. Add a silver wire (pseudo-reference electrode) and a platinum flag (counter electrode) to an oven-dried electrochemical cell.
4. Insert a freshly polished platinum button (2 mm^2 diameter) for use as the working electrode. Ensure that the bottom of the platinum button electrode is not touching the bottom of the electrochemical cell.

5. Fill the electrochemical cell with enough monomer electrolyte solution to ensure that the tips of all three electrodes are immersed in the solution.
6. De-aerate the solution for 5 min by gently bubbling argon through a needle immersed in the solution.
7. Raise the needle 2 mm above the solution and continue argon flow throughout the experiment to maintain an argon blanket over the solution.
8. Connect the electrodes to the potentiostat and begin the polymerization by cycling the applied potential five times at a sweep rate of 100 mV/sec and a potential range between -1.5 V and +1.0 V.
9. Record the current output during this process to generate cyclic voltammograms.

2. Polymer Electrochemistry
    1. After the polymer film is deposited on the platinum button working electrode, remove all the electrodes from the monomer electrolyte solution and gently rinse with monomer-free electrolyte solution (3 ml).
    2. Add the electrodes to a clean electrochemical cell and add enough monomer-free electrolyte solution to ensure that the tips of all three electrodes are immersed in the solution.
    3. Connect the electrodes to the potentiostat. Cycle the applied potential two times at a sweep rate of 50 mV/sec and a potential range between -1.5 V and +1.0 V.
    4. Repeat the experiment at 100, 200, 300, and 400 mV/sec. Record the current output during each experiment to generate cyclic voltammograms.

3. Preparation of Electropolymerized Films for UV-Vis-NIR Spectroscopy and Photothermal Studies
    1. Prepare polymer films as described in section 2.1 above, this time using an indium tin oxide (ITO)-coated glass slide as the working electrode. Grow the polymer films over 5 cycles at a scan rate of 100 mV/sec.
    2. After polymer deposition, remove the electrodes from the monomer solution and rinse with acetonitrile (5 ml).
    3. Store the polymer film in acetonitrile prior to spectroscopic studies.

3. NP Preparation

Figure 2 shows a schematic of the process used for NP preparation via emulsion polymerization.

1. Prepare a 1 ml solution of 2% (w/v) poly(4-styrenesulfonic acid-co-maleic acid) (PSS-co-MA) in water in a glass vial. Add a small magnetic stir bar to the vial. This is the aqueous phase.
2. Prepare 100 µl of 16 mg/ml monomer solution in chloroform in a microcentrifuge tube.
3. Prepare the organic solution by dissolving 0.03 g of dodecylbenzene sulfonic acid (DBSA) in the 100 µl monomer solution. Mix the organic solution using an automatic vortex mixer for 30-60 min in order to ensure homogeneity of the solution.
4. Add the organic phase to the aqueous phase dropwise in 10 µl portions while stirring with a magnetic stir bar until the complete volume of the organic solution is used. Allow stirring for 60 sec in between additions.
5. Add 2 ml of water to dilute the mixture. Remove the stir bar from the vial.
6. Sonicate the emulsion using a probe sonicator for a total of 20 sec in 10-sec intervals at an amplitude of 30% while immersing the vial in an ice bath.
7. Remove the sample vial from the ice bath, replace the stir bar, and continue stirring the emulsion.
8. Add 3.8 µl of 100 mg/ml solution of $FeCl_3$ in water to the monomer emulsion. Allow the polymerization to occur for 1 hr while continuously stirring. This protocol yields NPs of polymer stabilized with PSS-co-MA.
9. Remove the NP suspension from the stir plate and transfer into 7 ml centrifuge tubes. Centrifuge the suspension at 75,600 x g for 3 min; recover the supernatant and discard pellet.
10. Dialyze the supernatant for 24 hr using 100 kDa molecular weight cutoff (MWCO) dialysis tubing.

4. Polymer Films and NP Characterization

Note: Characterize the polymer films and NPs via UV-Vis-NIR spectroscopy, and the NPs using dynamic light scattering, zeta potential analysis, and electron microscopy.

1. Determination of Polymer Absorption in the UV-Vis-NIR Spectrum[29]
    1. NP suspensions: Transfer the suspension to a quartz cuvette and acquire a spectrum from 300 – 1,000 nm at a scan interval of 5 nm.
    2. Oxidized polymer films: Transfer the polymer-coated ITO glass slide to a quartz cuvette and fill the cuvette with anhydrous acetonitrile. Add 2 drops of a 100 mg/ml solution of $FeCl_3$ in $CHCl_3$ to the acetonitrile and mix to ensure the polymer film is fully oxidized. Acquire a spectrum from 300 – 1,000 nm at a scan interval of 5 nm.
    3. Reduced polymer films: Transfer the polymer-coated ITO glass slide to a cuvette and fill the cuvette with anhydrous acetonitrile. Add one drop of hydrazine to the liquid and mix to ensure the polymer film is fully reduced. Acquire a spectrum from 300 – 1,000 nm at a scan interval of 5 nm.

2. Determination of NP Size Using Dynamic Light Scattering (DLS)[30]
    1. Turn on the DLS instrument and allow it to warm up for 15 min.
    2. Dilute the NP suspension in water to a concentration of 0.01 mg/ml and place in a disposable polystyrene cuvette.
    3. Place cuvette in reader and begin measurement.

3. Determination of NP Zeta Potential[31]
    1. Turn on the zeta potential instrument and allow it to warm up for 30 min.
    2. Prepare the sample by diluting 200 µl of NP suspension in 800 µl of 10 mM KCl solution.
    3. Fill a disposable polystyrene cuvette with 700 µl of the sample.

4. Insert the zeta potential electrode cell into the sample ensuring that no bubbles are trapped between the electrodes or in the laser light path.
5. Insert the cuvette in the instrument and follow software instructions for running the measurement.

4. Determination of NP Size Using Scanning Electron Microscopy (SEM)[32]
    1. Drop-cast 10 µl of the NP suspensions onto Si wafers and allow to dry.
    2. Sputter coat the dried NPs with 2 nm of iridium.
    3. Image the samples at a working distance of 5 mm and at 5 kV.

5. Investigate the Cytocompatibility of the NPs

Note: All cell manipulations should be carried out in a biosafety cabinet (laminar flow hood) to prevent contamination of the cells with bacteria, yeast, or fungi from the environment, and to protect the user from potentially infectious diseases. All solutions and supplies used with the cells should be sterile. Use proper aseptic cell culture techniques.

1. Culture the SKOV-3 ovarian cancer cells in T75 flasks at 37 °C in a CO_2 incubator (5% CO_2) using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum as growth medium.
2. Seed cells at a cell density of 5,000 cells/well in a 96-well plate and incubate for 24 hr at 37 °C in a CO_2 incubator.
3. Immediately before use, dilute NP suspension in full growth medium at a concentration of 1 mg/ml.
4. Filter the NP suspensions by passing through a sterile 0.2-µm filter and dilute to the desired exposure concentrations (2–500 µg/ml) with full growth medium supplemented with 1% penicillin/streptomycin.
5. Remove the media from each of the wells in the 96-well plate by gently pipetting and replace with 100 µl of NP suspensions at the various exposure concentrations, or with 100 µl of NP-free media for both positive and negative cytocompatibility controls. Utilize 6 replicate wells per condition.
6. Immediately before the next step, prepare a 0.5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phenol red-free DMEM. Sterile filter the MTT solution through a sterile 0.2-µm filter.
7. After allowing the NPs to incubate with the cells for the desired period of time (typically 24 or 48 hr), remove NP suspensions by carefully pipetting out.
8. Immediately replace the media with the following depending on the condition:
    1. For the negative cytocompatibility control, add 100 µl methanol to each of 6 wells and allow to sit for at least 5 min. After methanol treatment, replace the methanol with 100 µl of sterile-filtered 0.5 mg/ml MTT solution in phenol red-free DMEM.
    2. For the positive control and NP-treated samples, replace the medium with 100 µl of sterile-filtered 0.5 mg/ml MTT solution in phenol red-free DMEM.
9. Incubate the cells for 2 to 4 hr in the incubator. After incubation, examine the cells under the microscope to check for the formation of formazan crystals.
10. Carefully remove the MTT solution by pipetting and replace it with 100 µl of dimethylsulfoxide (DMSO).
11. Place the 96 well plate on a shaker and mix for several minutes to encourage dissolution of the formazan crystals.
12. Measure the absorbance of each well at 590 nm (peak absorbance of formazan product) and 700 nm (baseline).
13. Subtract the sample absorbance at 700 nm (baseline) from that at 590 nm for each well.
14. Normalize the corrected absorbance by dividing it by the average of the positive control and convert to a percentage by multiplying by 100.
15. Determine the average percent viability and standard deviation for each condition.

6. Photothermal Transduction Studies

Note: In this work a laser system previously described by Pattani and Tunell is utilized.[33]

1. Photothermal Transduction of NP Suspensions
    1. Dilute NPs in DI water to the concentration of interest.
    2. Add 100 µl of NP suspension to a well of a 96-well plate. Place the well plate on a hot plate maintained at 25 °C.
    3. Turn on the power supply to the laser and allow it to warm for several minutes. In this study a fiber-coupled 808-nm laser diode rated up to 1 W of power is used.
    4. Route the laser beam toward the sample stage via an optical fiber. Use a convex lens to diverge the laser beam to the desired spot size.
    5. Measure the power output using a standard power meter and adjust to a power of 1 W/cm^2.
    6. Turn on IR camera (InSb infrared camera (FLIR Systems SC4000)) and set the region of interest (ROI) spot to read the temperature of the 6 mm spot where the laser is focused.
    7. Place the well of interest at the focal point of the laser beam. Record the baseline temperature of the sample. Turn on the laser and irradiate the well continuously for 5 min while recording the temperature.
    8. After 5 min, turn off the laser and continue recording the temperature of the well until it cools back to the starting baseline temperature. Note: Heat and cool each suspension three times and calculate the average temperature change over time. Use DI water at 25 °C instead of a NP suspension as a negative control for photothermal conversion.

2. Photothermal Transduction of Polymer Films
    1. Transfer the polymer-coated ITO glass slide to a hot plate maintained at 25 °C.
    2. Turn on the power supply to the laser and allow it to warm for several minutes. In this study a fiber-coupled 808-nm laser diode rated up to 1 W of power is used.

3. Route the laser beam toward the sample stage via an optical fiber. Use a convex lens to diverge the laser beam to the desired spot size.
4. Measure the power output using a standard power meter and adjust to a power of 1 W/cm^2.
5. Turn on IR camera (InSb infrared camera (FLIR Systems SC4000)) and set the region of interest (ROI) spot to read the temperature of the 6 mm spot where the laser is focused.
6. Place the film at the focal point of the laser beam. Record the baseline temperature of the sample. Turn on the laser and irradiate the sample continuously for 5 min while recording the temperature.
7. After 5 min, turn off the laser and continue recording the temperature of the sample until it cools back to the starting baseline temperature.
   Note: Heat and cool each film three times and calculate the average temperature change over time. Use a bare ITO slide at 25 °C as a negative control for photothermal conversion.

Representative Results

The reaction protocol yielding M1 and M2 is shown in **Figure 1**. The monomers can be characterized by ^{1}H and ^{13}C NMR spectroscopy, melting point, and elemental analysis. The ^{1}H NMR spectrum provides information regarding the connectivity of atoms and their electronic environments; thus, it is routinely used to verify that reactions have been completed successfully. Negishi coupling reactions involve coupling of the phenyl ring to the EDOT, causing the phenyl proton peak to shift from 7.1 ppm to 7.8 ppm. The thienyl proton will also shift upfield to 6.5 ppm. The four protons on the ethylenedioxy bridge carbons will split into two sets of multiplets at 4.3 ppm. Protons on aliphatic carbons will not change significantly. The ^{13}C NMR spectrum will exhibit peaks at 170, 145, 140, and 113 for the thienyl carbons, and 150, 120, and 112 for the phenylene carbons. Positions of aliphatic carbons will not change significantly. The chemical structure, ^{1}H NMR, and ^{13}C NMR of M2 are shown in **Figure 3**.

Electropolymerizations of M2 yielding polymer (P2) and cyclic voltammetry of P2 are shown in **Figure 4**. In **Figure 4A**, initially, there is no current response; as potential increases, the onset of oxidation of the M1 monomer ($E_{on,m}$) can be seen at + 0.25 V, with the peak oxidation of the monomer ($E_{p,m}$) at + 0.61 V. During the first scan, the initial peak observed is indicative of irreversible monomer oxidation, resulting in P2 formation on the surface of the working electrode. During the second scan two oxidation processes are observed: monomer oxidation is still seen at +0.25 V, and polymer oxidation is seen at 0 V. Cyclic voltammetry of P2 (**Figure 4B**) was conducted at scan rates ranging from 50 to 400 mV/sec. The polymer film is dark blue in the oxidized state and red in the neutral state. Cycling the polymer at a variety of scan rates reveals a linear relationship between scan rate and peak current, indicating that the polymer is electroactive and adhered to the electrode.[18] Polymer oxidation ($E_{a,p}$) is observed at -0.02 V for P2, and polymer reduction ($E_{c,p}$) is observed at -0.3 V when cycled at 100 mV/sec.

The NPs were synthesized as shown in **Figure 2** and characterized using UV-Vis-NIR spectroscopy, electron microscopy, and DLS. The UV-Vis-NIR spectra of oxidized and reduced P2 films, and of oxidized P2 NPs, are shown in **Figure 5**. The oxidized polymer films and NPs exhibit a peak absorbance λ_{max} at 1.56 eV (795 nm). When reduced in hydrazine, the film peak absorbance shifts to a λ_{max} of 2.3 eV (540 nm). The polymer band gap (E_g) is determined from the onset of the π- π* transition in the neutral polymer, as indicated by the black arrow in **Figure 5**.

The SEM image of P2 NPs in **Figure 6A** shows that the NPs are spherical and sub-100 nm in diameter. DLS data in **Figure 6B** shows a Z-average of the suspensions to be 104 nm in diameter with a polydispersity index (PDI) of 0.13, indicating that the sample is moderately monodispersed. The zeta potential of the P2 NPs was found to be -30.5 mV. Change in temperature when NPs are exposed to NIR radiation demonstrates photothermal conversion. Compared to water controls, which undergo less than a 1 °C increase in temperature, NP suspensions in water are able to convert the absorbed laser energy into heat as demonstrated by the 30 °C increase in temperature of the NP suspensions (**Figure 6C**). A similar temperature increase (28 °C) is observed when polymer films on ITO glass are irradiated at 808 nm (**Figure 6C**).

The cytocompatibility of polymer NPs is determined using MTT cell viability assays. Results of cytocompatibility studies for PEDOT:PSS-co-MA NPs are shown in **Figure 7**. As shown, within the NP concentration range of 0.23 to 56 µg/ml, the NPs do not decrease cell viability to less than 90% of control. Typically, a reduction in cell viability of less than 20% (*i.e.,* up to 80% viability) is considered acceptable for determination of NP cytocompatibility.

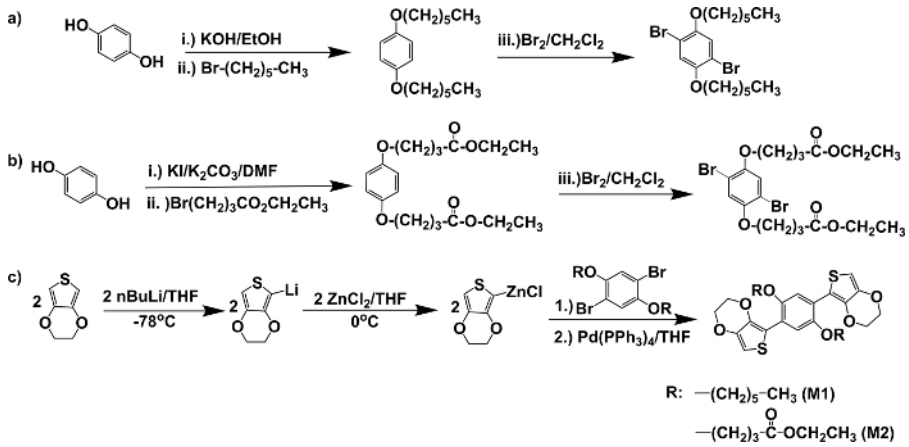

Figure 1. General monomer synthesis starting with the precursor synthesis. (**A**) Synthesis of 1,4-dialkoxy-2,5-dibromobenzene. (**B**) Synthesis of 1,4-dialkoxy-2,5-dibromobenzene containing ester moiety. (**C**) Cross-coupling reaction of 1,4-dialkoxy-2,5-dibromobenzene with EDOT, yielding monomers M1 and M2. Please click here to view a larger version of this figure.

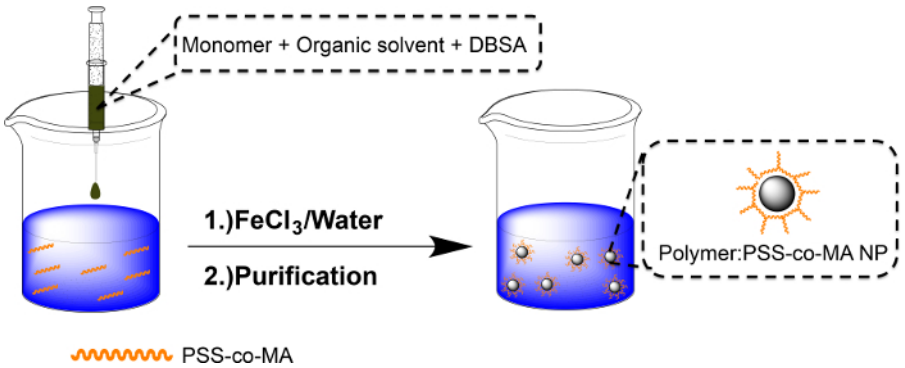

Figure 2. Polymerization process in which the organic solution is added dropwise to an aqueous solution creating an emulsion. The monomer and the organic solvent may vary. Oxidative polymerization occurs when $FeCl_3$ is added to the emulsion. After purification of the colloidal suspension, the NPs are suspended in the aqueous medium. Please click here to view a larger version of this figure.

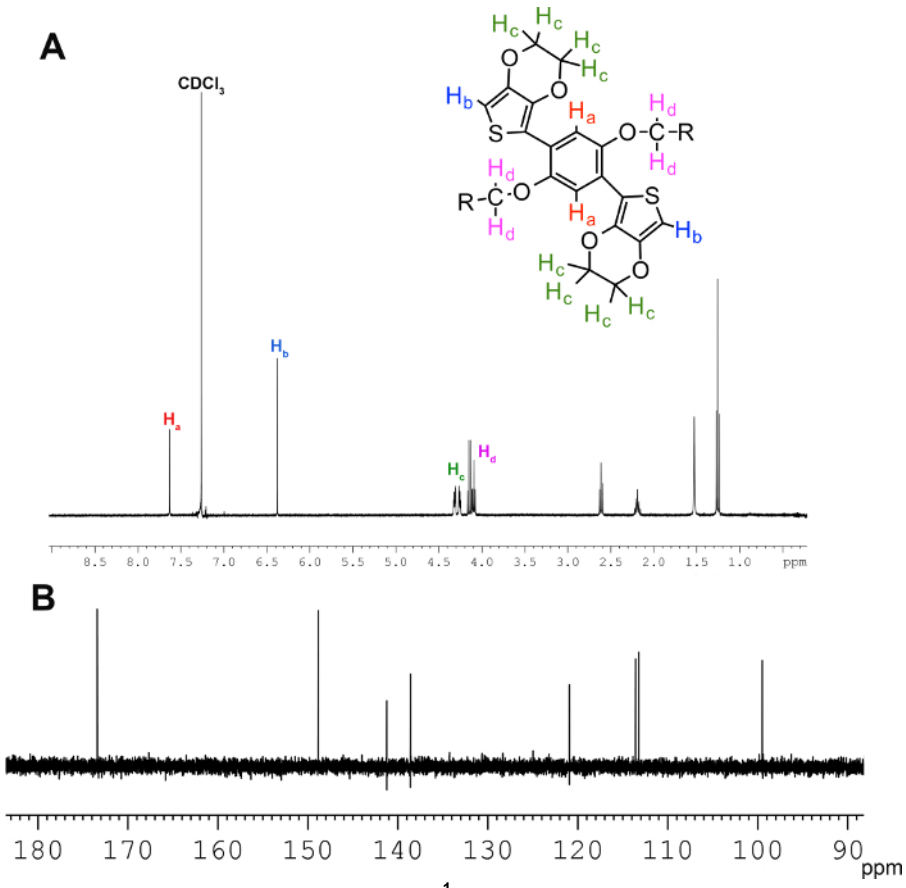

Figure 3. NMR spectra of monomer M2. (**A**) ^{1}H NMR spectroscopy of M2 where the splitting of the ethylenedioxy protons at 4.32 ppm, the upfield shift of the thienyl protons, and the upfield shift of the phenyl protons are indicative of successful coupling. (**B**) ^{13}C NMR spectroscopy of M2 showing the thienyl and phenyl carbon peaks. Please click here to view a larger version of this figure.

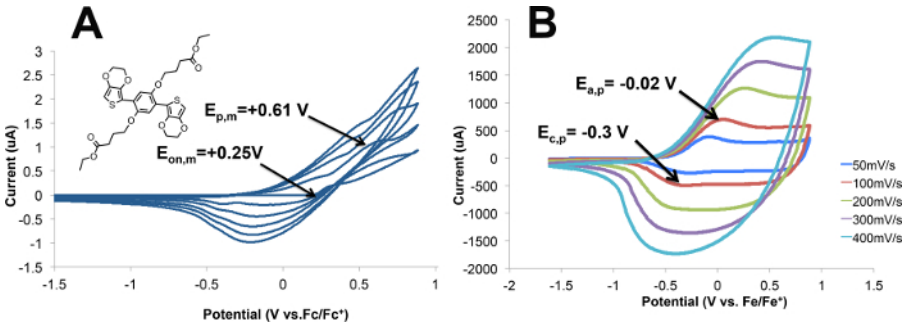

Figure 4. (**A**) Electrochemical polymerization of M2 to P2; five cycles at 100 mV/sec of 0.01 M M2 in 0.1 M TBAP/CH_3CN. (**B**) Cyclic voltammetry of the polymer film in 0.1 M TBAP/CH_3CN cycled at 50, 100, 200, 300, and 400 mV/sec. Please click here to view a larger version of this figure.

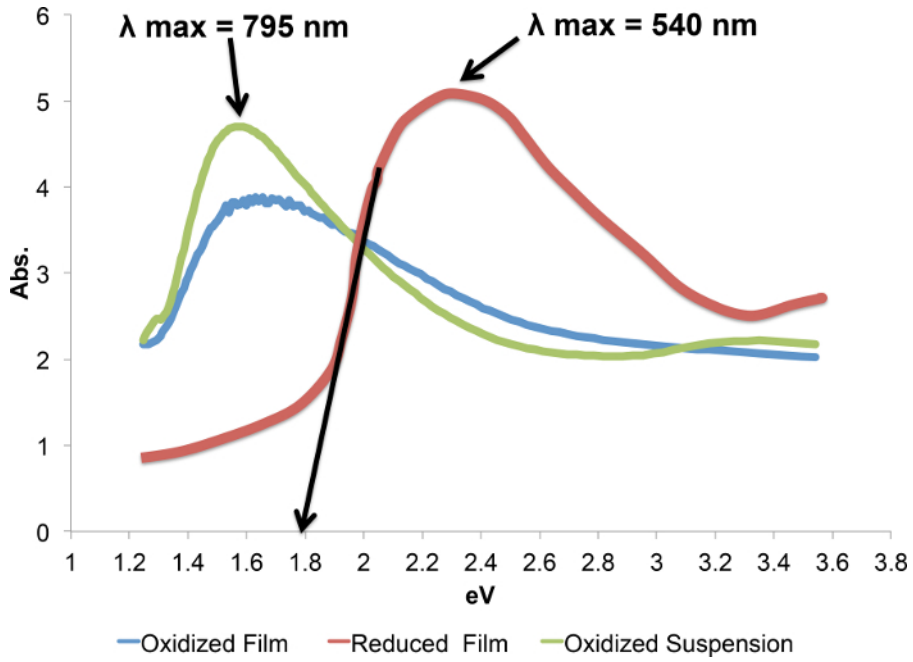

Figure 5. UV-Vis-NIR spectra of P2 both as a film and as a suspension of NPs. The spectrum of the oxidized film is shown in blue, the spectrum of the reduced film is shown in red, and the spectrum of the oxidized NP suspension is shown in green. The black arrow corresponds to the tangent line used for determination of the polymer bandgap. Peak absorption wavelengths for the polymers are provided. Please click here to view a larger version of this figure.

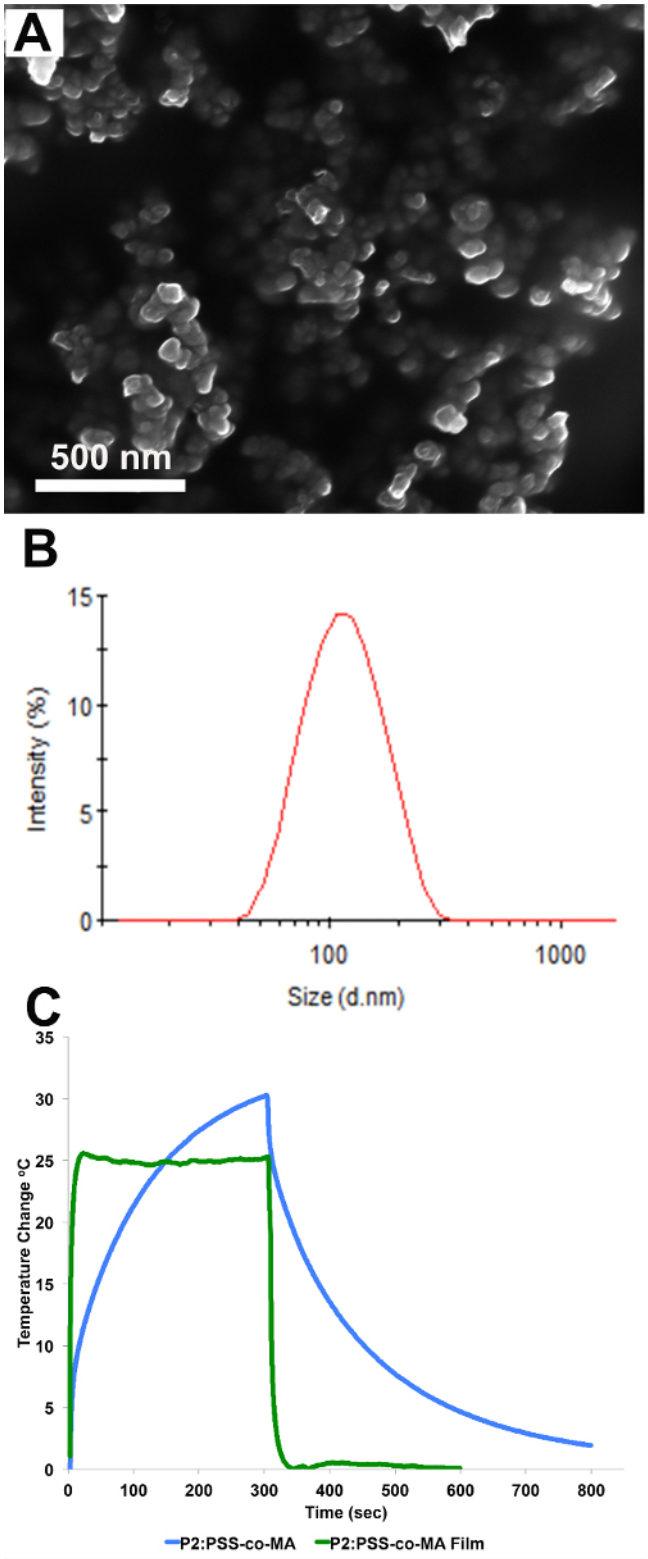

Figure 6. (**A**) SEM image showing the morphology and size of P2 NPs. (**B**) Size distribution of P2:PSS-co-MA NP suspension where the Z-average value is 104 nm and the PDI is 0.13. (**C**) Temperature change of a P2:PSS-co-MA NP suspension at 1 mg/ml (blue) and film (green) when irradiated with NIR light for 300 sec, followed by passive cooling upon completion of laser irradiation. Please click here to view a larger version of this figure.

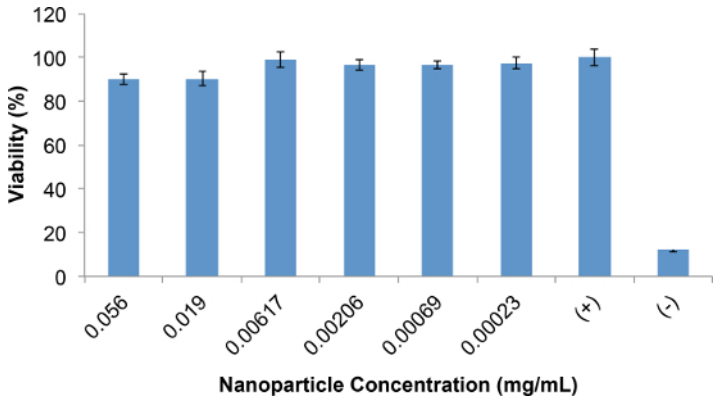

Figure 7. Cytocompatibility of PEDOT:PSS-co-MA NP suspensions as determined by the MTT assay. Viability is shown for cells exposed to varying concentrations of NPs as the average percentage relative to that of cells incubated with NP-free media (positive control). Negative control consists of cells killed by exposure to methanol prior to the MTT assay. Error bars represent the standard deviation between replicates (n = 6). Please click here to view a larger version of this figure.

Discussion

In this work, electroactive polymer NPs have been synthesized as potential PTT agents for cancer treatment. The preparation of the NPs is described, starting with the synthesis of the monomers followed by emulsion polymerization. While the preparation of NPs using electroactive polymers such as EDOT and pyrrole has been described before, this paper describes the preparation of polymeric NPs starting with unique extended conjugation monomers, demonstrating that this process can be extended to larger, more complex monomers.

Two different routes are necessary to synthesize the dialkoxybenzene monomers. While the 1,4-dihexyloxybenzene can be synthesized using KOH/EtOH, that approach is unsuccessful in the synthesis of 1,4-bis(ethyl butanoyloxy)benzene, most likely due to base-promoted ester hydrolysis. When a KI/K_2CO_3 mixture is used, hydrolysis is avoided, and the product is successfully obtained. Bromination of both dialkoxybenzenes is accomplished using Br_2. It is necessary to conduct this experiment under flowing argon to displace HBr formed during the reaction. The gas outlet should vent over a neutralizing NaOH solution to prevent HBr from corroding hood fixtures; note that HBr may cause plastic tubing to harden over time.

BEDOT-$B(OR)_2$ monomers M1 and M2 were synthesized using Negishi coupling. This is an effective method for the carbon-carbon coupling of EDOT with 1,4-dialkoxy-2,5-dibromobenzenes to yield BEDOT-$B(OR)_2$ monomers. It is crucial to chill the EDOT to -78 °C prior to the addition of nBuLi, in order to minimize undesirable side reactions. When all the 1,4-dialkoxy-2,5-dibromobenzene is depleted from the reaction mixture (determined using TLC; this typically takes 3-5 days), the reaction is complete. The reaction is extremely air sensitive, and any exposure to air will affect the yield of the reaction. Thus, when introducing solid compounds (such as the catalyst) into the sealed flask, air exposure should be minimized by increasing argon flow.

Electroactive monomers and polymers are routinely characterized using cyclic voltammetry to determine monomer and polymer oxidation potentials and polymer reduction potentials, and films prepared via electrochemical polymerization are used to determine polymer absorption in the UV-Vis-nIR spectrum in both the oxidized and reduced states. In this work, polymer films were deposited onto both a platinum button and ITO coated glass by electropolymerization. Some of the advantages of electropolymerization are reproducibility and the ability to control the film thickness by monitoring the current of the polymerized film and stopping the electropolymerization when a specific response is achieved.[34] Electrochemical experiments must be conducted under an inert atmosphere such as argon; the argon flow should be so slow as to not disturb the surface of the solution to ensure a diffusion-controlled process. Alternatively, the electrochemical experiments can be performed in an inert atmosphere dry box fitted with electrochemical feedthroughs. It is important that none of the three electrodes are touching each other during electropolymerization. Prior to polymer cyclic voltammetry studies, the deposited polymer films must be washed with monomer-free electrolyte solution to remove any unreacted monomer from the films. For all electrochemical studies the potential range needed will depend on the structure of the monomer/polymer; so this range may vary with alternative monomers and polymers. Depending on the structure of the alkoxy substituents, the solvent used to prepare the monomer electrolyte solutions may also dissolve the polymer. In that case, polymer deposition on the electrode during electropolymerization will be slow or non-existent, and the solvent used for polymerization must be changed.

Emulsion polymerization for the preparation of NPs composed of electroactive polymers is an effective method that yields NPs with a uniform morphology. In this work, the emulsion polymerization process utilizes the same oxidative polymerization mechanism utilized during electrochemical polymerization; the major difference is that a chemical oxidant (ferric chloride) is used instead of an applied electrochemical potential. This emulsion polymerization, therefore, produces NPs identical in chemical composition to the films prepared via electrochemical polymerization. While electrochemical polymerization provides a facile means of characterizing the redox properties of the monomers and polymers, emulsion polymerization is a quick, inexpensive, and reproducible process that is easily scalable and can potentially be used with a number of different electroactive polymers. Emulsion polymerization also enables the preparation of NPs from polymers that have low solubility in organic and aqueous solutions that could not be emulsified effectively from the polymeric state. In our emulsion polymerizations, the organic phase was comprised of monomer, organic solvent (hexane), and dodecylbenzene sulfonic acid (surfactant). The aqueous phase was comprised of water, ferric chloride (oxidant), and PSS-co-MA (surfactant). The emulsion polymerization process is preceded by a sonication step to ensure the organic phase is well dispersed in the aqueous phase. During sonication, it is necessary to immerse the emulsion in an ice bath to prevent bulk heating. The surfactants PSS-co-MA and DBSA enable the dispersion of the synthesized NPs in aqueous solutions through inter-particle electrostatic repulsive forces. These surfactants also act as additional charge-balancing dopants and have been shown to produce spherical

NP geometry.[24] The polymeric NPs remain in the oxidized state, (as evidenced by the peak absorption at 795 nm; **Figure 4**), which is critical for biomedical applications in which absorption in the NIR range is necessary.[24]

Zeta potential analysis is commonly performed to assess the stability of NP suspensions. Zeta potential is the potential at the boundary between the Stern layer where ions are strongly associated with the NP surface, and the diffuse layer where ions no longer interact with the NP surface.[31] Zeta potential measurements rely on the movement of charged NPs when an electric field is applied to the suspension. Specifically, negatively charged NPs are attracted toward the positive electrode, and vice versa. Colloidal suspensions can be stabilized via electrostatic repulsions. Specifically, suspensions are considered stable when their zeta potential is greater than +/- 30 mV. In our NP formulations, the presence of sulfonate and carboxylate groups from DBSA and PSS-co-MA yields a negative surface charge on the NPs.

Purification of the NPs is a crucial step in order to remove any excess surfactant and any unreacted starting material prior to *in vitro* cell studies. Ineffective surfactant removal can lead to significant cell death. As for any other *in vitro* cell assay, it is vital to work in a laminar flow hood and to work under sterile conditions. NPs should also be sterilized prior to use by passing the suspension through a sterile 0.2-µm filter. It is also important to verify the concentration of NP suspensions after sterile filtering. For this purpose, a fraction of the filtered NP suspension of known volume can be freeze dried to obtain the dry mass. The MTT cell viability assay is typically used to study the effect of biomaterials, including NPs, on cultured cells. This simple assay can be adapted to the investigation of the cytocompatibility of NP suspensions with any mammalian cell line. The MTT colorimetric assay is based on the conversion of a yellow tetrazolium dye into purple, insoluble formazan crystals which can then be dissolved in DMSO or acidic alcohol solutions.[35,36] When performing *in vitro* cell assays such as the MTT cell viability assay in multi-well plates, consistency in cell seeding and manipulation is critical to achieve minimal differences between replicate samples. Prior to and during the experiment, the seeded cells should be examined under a microscope to ensure consistent seeding and growth, and also to rule out any contamination. Finally, microscopy can also be utilized to confirm complete dissolution of formazan crystals after addition of DMSO.

Photothermal studies were conducted using a continuous laser at 808-nm. The use of continuous vs. pulsed lasers can heat materials differently. Previous studies have compared photothermal conversion and photothermal ablation with gold nanostructures as PTT agents,[37] but more research is needed to investigate photothermal conversion from polymeric NPs like the ones described herein. In this work, the laser was diverged into a convex lens and focused at a 6 mm spot size. It is important to be careful not to disturb the optical system when running experiments to prevent accidental changes in the focal plane that would cause differences in the photothermal conversion results. A hot plate was used to warm and maintain a constant baseline temperature for the study.

In conclusion, a protocol for NP preparation of electroactive polymers suspended in aqueous medium is described. Negishi coupling is an effective method for coupling 1,4-dialkoxy-2,5-dibromobenzenes with 3,4-ethylenedioxythiophene (EDOT). Electropolymerization of the monomers is detailed in this protocol. This proves to be an effective way to rapidly produce polymer films and study their electronic properties. The polymer films are further characterized using UV-Vis-NIR spectroscopy to determine the band gaps of the neutral polymers. Electrochemical emulsion polymerization yields sub-100 nm NPs with uniform spherical morphologies. In addition to photothermal ablation therapy, these NPs have many potential applications in electroactive devices, including energy storage and sensors. The thermal and cytocompatibility studies performed indicate that these NPs could be potential candidates in biomedical applications as photothermal agents.

Disclosures

The authors have nothing to disclose.

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